

THE COVALENT STRUCTURE OF MITOCHONDRIAL
ASPARTATE AMINOTRANSFERASE FROM CHICKEN.
IDENTIFICATION OF SEGMENTS OF THE
POLYPEPTIDE CHAIN INVARIANT SPECIFICALLY IN
THE MITOCHONDRIAL ISOENZYME

INAUGURAL - DISSERTATION

ZUR

ERLANGUNG DER PHILOSOPHISCHEN DOKTORWÜRDE
VORGELEGT DER

PHILOSOPHISCHEN FAKULTÄT II

DER

UNIVERSITÄT ZÜRICH

VON

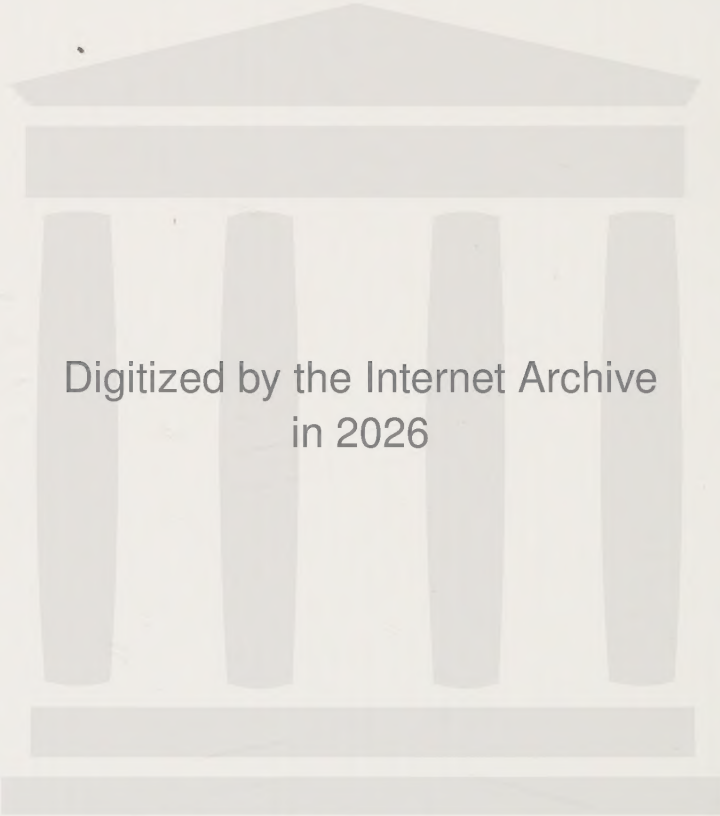
Ursula Graf-Hausner
aus Bäretswil / ZH

Begutachtet von Herrn
Prof. Dr. Ph. Christen

Zürich 1981

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DANK

Meinem Doktorvater Prof. Ph. Christen danke ich herzlich, dass ich unter seiner Leitung diese Arbeit ausführen durfte und dass er mich dabei so tatkräftig unterstützt hat. Mein Dank gebührt ebenfalls Herrn Dr. K. Wilson, der nicht nur zusammen mit seinem Laboranten Roland Stötzel einen Grossteil der chymotryptischen und besonders der tryptischen Peptide sequenziert hat, sondern der mir auch viele Male den Weg zu neuen Methoden geebnet hat. Den Herren Dr. G. Eichele und Prof. J. Janſonius danke ich für die gute Zusammenarbeit und für wertvolle Diskussionen im Zusammenhang mit der räumlichen Struktur des Proteins. Ich danke Frl. Katy Brunschweiler, die mir eine Zeitlang als Laborantin zur Seite stand und allen, die ebenfalls wertvolle Leistungen zu dieser Arbeit beigesteuert haben, so Frau Alice Gasser, die das Enzym präparierte, Frau Christina Kasper, die die Aminosäureanalysen ausführte, Herrn Hans-Peter Lutz, der den Flüssigphasensequenator wartete und Herrn Bas van Wieringen, der mir beim Bau des Modells behilflich war.



CONTENTS

SUMMARY	5
ZUSAMMENFASSUNG	6
INTRODUCTION	7
EXPERIMENTAL PROCEDURE	11
RESULTS	12
DISCUSSION	16
SUPPLEMENTARY MATERIAL	
1. Experimental procedure	29
2. Cyanogen bromide peptides	31
3. Chymotryptic peptides	38
4. Tryptic peptides	45
5. Peptides from digestion with <u>Staphylococcus aureus</u> protease V8	57
6. Tryptic peptides of N-2,3- $[^{14}\text{C}]$ ethylmaleimide- modified mAATase	60
REFERENCES	63

The abbreviations used are:

PLP, pyridoxal-5'-phosphate,

AATase, aspartate aminotransferase,

mAATase, mitochondrial aspartate aminotransferase,

cAATase, cytosolic aspartate aminotransferase,

HPLC, high performance liquid chromatography.

SUMMARY

The primary structure of mitochondrial aspartate aminotransferase from chicken is reported. The enzyme is a dimer of identical subunits. One subunit contains 401 amino acid residues, the calculated subunit molecular weight of the apoform is 44.866. The degree of sequence identity with the homologous cytosolic isoenzyme from chicken is 46%.

A comparison of the primary structures of the mitochondrial and the cytosolic isoenzyme from pig and chicken shows that 40% of all residues are invariant. Most of these residues are located in the active site cleft and in the interior of the molecule; apparently, they contribute to functionally important structures common to both isoenzymes. The degree of interspecies sequence identity both of the mitochondrial and the cytosolic isoenzyme from chicken and pig (86% and 83%, respectively) markedly exceeds that of the intraspecies identity between mitochondrial and cytosolic aspartate aminotransferase in chicken (46%) or in pig (48%). Based on these values the duplication of the aspartate aminotransferase ancestor gene is estimated to have occurred approximately 1000 million years ago, i. e., at the time of the emergence of eucaryotic cells.

By sequence comparison it is possible to identify amino acid residues and segments of the polypeptide chain that have behaved conservatively specifically in the mitochondrial isoenzyme during phylogenetic evolution. These segments comprise 38% of the total polypeptide chain and appear to cluster in a certain surface region. The cluster carries an excess of positively charged residues which exceeds the overall charge difference between the cytosolic ($pI \sim 6$) and the mitochondrial isoenzyme ($pI \sim 9$).

This work is part of an investigation of the structure and function of mitochondrial aspartate aminotransferase, a joint project with the protein crystallographer Prof. J. N. Jansonius and colleagues, University of Basel.

ZUSAMMENFASSUNG

In dieser Arbeit wird die Primärstruktur der mitochondrialen Aspartat-Aminotransferase des Huhns vorgestellt. Das Enzym ist ein Dimer aus identischen Untereinheiten. Eine Untereinheit enthält 401 Aminosäurereste, das berechnete Molekulargewicht der Apoform beträgt 44'866. Der Grad an Sequenzidentität mit dem homologen cytosolischen Isoenzym des Huhns beläuft sich auf 46%.

Ein Vergleich der Primärstrukturen des mitochondrialen und des cytosolischen Isoenzym vom Schwein und vom Huhn zeigt, dass 40% aller Aminosäurereste in allen vier Proteinen invariant sind. Die meisten dieser Reste sind in der Tasche der aktiven Stelle und im Inneren des Moleküls lokalisiert; offensichtlich sind sie Teil funktionell wichtiger Strukturen, die beiden Isoenzymen gemeinsam sind. Die Inter-Spezies-Sequenzidentität sowohl des mitochondrialen Isoenzym des Huhns und des Schweins als auch diejenige des cytosolischen Isoenzym der beiden Spezies (86%, bzw. 83%) übersteigt deutlich die Intra-Spezies-Sequenzidentität zwischen mitochondrialer und cytosolischer Aspartat-Aminotransferase des Huhns (46%) oder des Schweins (48%). Aus den Zahlenwerten lässt sich abschätzen, dass sich die Verdoppelung des Stammens der Aspartat-Aminotransferase vor ungefähr 1000 Millionen Jahren ereignet hat, d.h. zu der Zeit, als eukaryontische Zellen aufgetreten sind.

Ein Sequenzvergleich ermöglichte, Aminosäurereste und Segmente der Polypeptidkette zu identifizieren, die sich während der phylogenetischen Evolution spezifisch im mitochondrialen Isoenzym konservativ verhalten haben. Diese Segmente umfassen 38% der gesamten Polypeptidkette und häufen sich in einem bestimmten Oberflächenbezirk. Die Segmente tragen einen Ueberschuss an positiv geladenen Aminosäureresten, der grösser ist als die gesamte Ladungsdifferenz zwischen dem cytosolischen ($pI \sim 6$) und dem mitochondrialen Isoenzym ($pI \sim 9$).

Diese Arbeit ist Teil eines Forschungsprojektes über die Struktur und Funktion der mitochondrialen Aspartat-Aminotransferase, das gemeinsam mit dem Proteinkristallographen J. N. Jansonius und seiner Gruppe (Universität Basel) durchgeführt wird.

INTRODUCTION

Numerous reactions in the metabolism of amino acids are catalyzed by enzymes containing pyridoxal-5'-phosphate (PLP) as cofactor. PLP (I) is the biologically active form of vitamin B₆ (pyridoxine). All PLP-dependent enzymic reactions start with a common intermediate, in which the amino acid substrate is linked to the coenzyme by an azomethine linkage (II, external aldimine). The different reactions proceeding from this intermediate may be divided into three groups:

1. The cleavage of the bond between the α -carbon and the α -hydrogen atom of the substrate leads to a semiquinonoid intermediate (III), which may react in various ways:
 - a) In transamination a proton is added to the 4'-carbon atom. The resulting ketimine intermediate (IV) is hydrolyzed to an α -keto acid and pyridoxamine phosphate (V). In the second half-reaction the enzyme-pyridoxamine phosphate complex reacts with a second α -keto acid, to which the amino group is donated by reversal of the steps described above with regeneration of PLP. In this double displacement reaction the prosthetic group acts as a carrier of amino groups from an amino acid to a keto acid.
 - b) The non-stereospecific readdition of a proton to the α -carbon of the semiquinonoid intermediate (III) leads to racemization of the amino acid.
 - c) When a good leaving group is present in β -position of the amino acid, such as OH⁻ in the case of serine and threonine, it can be eliminated. This leads to the Schiff base of aminoacrylate or aminocrotonate, which can undergo hydrolysis to give the free aminoacrylate or aminocrotonate and PLP. After tautomerization to an imino acid and hydrolysis of the latter the final products are α -keto acid and ammonium ion.
2. The cleavage of the bond between the α -carbon atom and the carboxyl group is catalyzed by amino acid decarboxylases. This reaction also leads to a semiquinonoid intermediate (III). Addition of a proton at the site of decarboxylation and following hydrolysis of the Schiff base gives an amine and PLP.
3. In a third type of reaction the side chain of the external aldimine (II) undergoes an aldol cleavage.

Examples of PLP-dependent reactions are given in Table I.

The fact, that all PLP-dependent enzymes show extensive similarities in their coenzyme-substrate interactions but catalyze such varied reactions implies that the apoenzyme moiety not only determines the substrate specificity of the holoenzyme but also its reaction specificity.

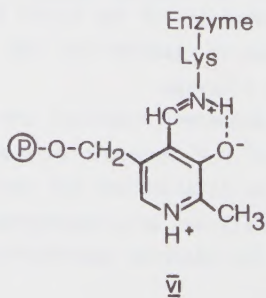
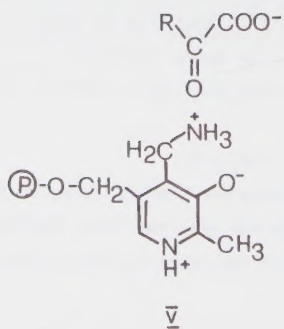
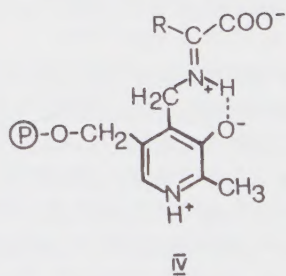
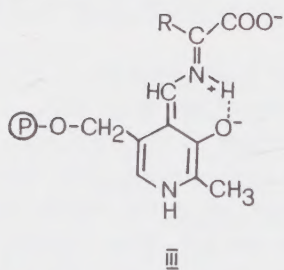
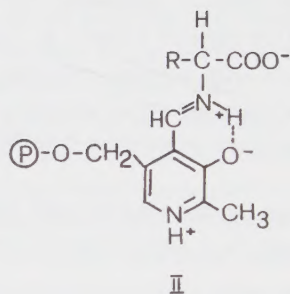
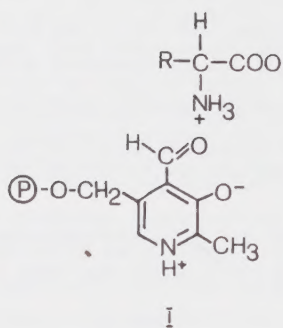
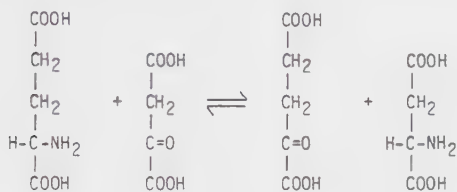
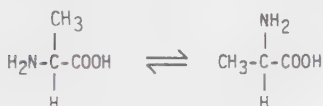


TABLE I - Pyridoxal-5'-phosphate dependent reactions

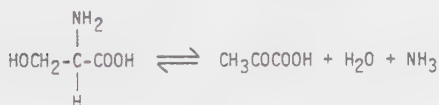
Transamination, e.g., aspartate aminotransferase



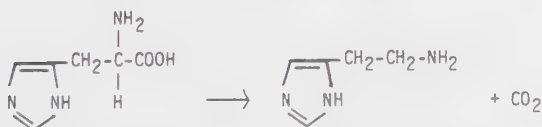
Racemisation, e.g., alanine racemase



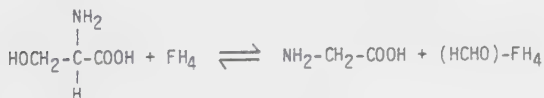
Elimination, e.g., serine dehydratase



Decarboxylation, e.g., histidine decarboxylase



Aldol cleavage, e.g., serine transhydroxymethylase



(FH₄, tetrahydrofolic acid)

The most extensively studied PLP-dependent enzyme is aspartate aminotransferase (AATase, also known as glutamate oxalacetate transaminase, GOT). As in all other PLP-dependent enzymes examined so far, the coenzyme is linked to the ϵ -amino group of a lysine residue by a Schiff base; this form is called the internal aldimine (VI).

Two genetically independent isoenzymes of AATase have been found in animal tissues and possibly occur in all eucaryotic cells. Both isoenzymes are composed of two identical polypeptide chains of about 400 amino acid residues. One isoenzyme is located in the mitochondrial matrix (mAATase) and the other in the cytosol (cAATase). Both are coded for by nuclear DNA. The mitochondrial isoenzyme is synthesized as a precursor of higher molecular weight (1) and is assumed to be translocated posttranslationally from its site of synthesis into the mitochondrial matrix (2). Neither isoenzyme seems to show organ- or tissue-specific properties (3-5). The essential features of their catalytic mechanisms are identical (6). The development of two independent gene products might relate to the independent regulation of their synthesis and degradation (7). The three-dimensional structure of chicken mAATase in the internal aldimine form (PLP enzyme) has been determined at 2.8 Å resolution (8), and the high-resolution X-ray analyses of cAATase from chicken (9) and pig (10) are underway. The amino acid sequences of the cytosolic and the mitochondrial isoenzyme from pig have been elucidated (11-15). A sequence identity of 48% indicates that they are homologous proteins. A comparison of the available amino acid sequence of the cytosolic isoenzyme from chicken (16) with that from pig has revealed a high degree of interspecies sequence identity (83%) which markedly exceeds that of the intraspecies identity (48%) of the cytosolic and mitochondrial isoenzyme from pig. Immunological cross-reactivity studies indicated an even closer interspecies similarity for the two mitochondrial isoenzymes (17).

In the present study, the complete amino acid sequence of mAATase from chicken is reported. The determination of the amino acid sequence of this protein as the last of the isoenzymes from chicken and pig offered the opportunity to identify structural features characteristic for this organelle-confined isoenzyme.

EXPERIMENTAL PROCEDURE

Mitochondrial AATase was isolated from chicken heart as described previously (18). The protein was fragmented and the peptides isolated as outlined below (for details, see Supplementary Material):

- (1) Dithiothreitol-reduced and carboxymethylated protein (iodo [^{14}C] acetic acid) was cleaved with cyanogen bromide. Ten CNBr peptides were isolated by Sephadex gel filtration, high voltage paper electrophoresis, and high performance liquid chromatography (HPLC) on a reverse phase C 18 column.
- (2) CNBr peptides from pool II and III eluted from a Sephadex G-75 column (Fig. 4) were subjected to chymotryptic digestion. By a variety of different techniques including column chromatography on a cation exchange resin M-72, high voltage electrophoresis, and thin-layer chromatography on cellulose 31 different chymotryptic peptides were isolated.
- (3) A restricted tryptic hydrolysis after citraconylation was carried out on the carboxymethylated protein and 27 peptides were isolated.
- (4) Aminoethylated protein was digested with Staphylococcus aureus protease V8 and 10 peptides were purified as indicated under (2) above.
- (5) In order to isolate the sulfhydryl peptides which could not be isolated otherwise, the protein was labeled with N- [^{14}C] ethylmaleimide and subjected to tryptic digestion. Four sulfhydryl peptides were isolated by diagonal high voltage electrophoresis (19).

The amino acid sequences of the peptides were determined by Edman degradation either with phenylisothiocyanate or 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (20, 21), or by digestions with exopeptidases. Larger peptides were first digested with endopeptidases and the resulting fragments separated and analyzed as indicated above. Asparagine and glutamine were determined by at least two of the following methods: electrophoretic mobility of the peptides at pH 6.5, direct identification of the thiohydantoin derivatives by HPLC, and amino acid analysis following hydrolysis with exopeptidases.

After determination of their amino acid sequence, the peptides were tentatively aligned on the basis of the sequence of mAATase from pig (13). Not all overlaps were determined. The electron density maps at 2.8 Å resolution allowed the unambiguous tracing of the polypeptide chain in which the amino acid sequence could be accommodated (8).

RESULTS

The polypeptide chain of mAATase from chicken contains 401 amino acid residues, i.e., 9 residues less than the cytosolic isoenzyme from chicken and pig. The complete amino acid sequence is presented in Fig. 1. The corresponding amino acid composition is Lys₂₈, His₉, Arg₂₄, Asp₂₀, Asn₁₇, Thr₁₇, Ser₂₉, Glu₂₅, Gln₁₃, Pro₁₇, Gly₃₀, Ala₃₆, Cys₅, Val₂₄, Met₁₃, Ile₂₄, Leu₃₂, Tyr₁₅, Phe₁₆, Trp₇. The subunit molecular weight of the apoenzyme calculated from this amino acid composition is 44.866. Values obtained by polyacrylamid gel electrophoresis in the presence of SDS (18) are in good agreement with this molecular weight. The protein does not contain detectable amounts of neutral sugars. Mitochondrial AATase and pure α -subform of cytosolic AATase from chicken (82 nmol each, isolated according to Ref. 18, 23), were dialyzed against 3 x 1000 volumes of water and analyzed for sugar by the orcin method of Winzler (24). Only 0.1 and 0.2 mol hexose per mol subunit were detected in mAATase and cAATase, respectively. These analyses are at variance with a previous report on the subforms of cytosolic isoenzyme from pig which were described to contain at least 1 mono- or oligosaccharide unit per peptide chain (25).

Following pages:

Fig. 1 - The complete amino acid sequence of mAATase from chicken. Notations indicate the peptides produced by different fragmentation procedures: CNBr, cyanogen bromide; C, chymotrypsin; T, trypsin; S, Staphylococcus aureus protease V8; NEM-T, peptides from tryptic digest of protein labeled with N- $[^{14}\text{C}]$ ethylmaleimide; $\longrightarrow$, Edman degradation of the total protein (see Supplementary Material Table XXVII); Pxy-Lys, pyridoxal-5'-phosphate binding peptide (22). The peptides, or parts of peptides which are marked with solid lines were sequenced. Segments marked with dashed lines were assigned on the basis of their amino acid composition. For the procedure in arranging the peptides, see Experimental Procedure. For analytical data on the individual fragments and for details of the experimental procedure, see Supplementary Material.

10 20 30 40 50
 S S W S H V E M G P P D P I L G V T E A F K R D T N S K K M N L G V G A Y R D D N G K P Y V L N C
 CNBr-1 CNBr-2 CNBr-3
 T-1 C-1 C-2 C-3
 S-1 NEM-T-1
 60 70 80 90 100
 V R K A E A M I A A K K M D K E Y L P I A G L A D F T R A S A E L A L G E N S E A F K S G R Y V T V
 CNBr-4 CNBr-5
 C-4 T-3 T-4 T-5 T-6 T-7
 110 120 130 140 150
 Q G I S G T S L R V G A N F L Q R F F K F S R D V Y L P K P S W G N H T P I F R D A G L E L Q A Y
 T-8 T-9 T-10 T-11 T-12
 160 170 180 190 200
 R Y Y D P K T C S L D F T G A M E D I S K I P E K S I I L L H A C A H N P T G V D P R E E Q W K E L
 T-13 T-14 T-15
 S-2 S-3 S-4
 NEM-T-2 NEM-T-3

ASVVKRNLLAYFDMAYQGASGDINRDAAWALRHFIEQGI DVVLSQSYAK
 220 230 240 250
 C-9 C-10 C-11 T-17 T-18 T-19 C-12
 T-16

pxy-

NMGLYGERAGFTVICRDAEEAKRVESQLKILIRPMYSNPPMNGARIASL
 260 270 280 290 300
 Lys CNBr-6 C-13 C-14 C-15 C-16 C-17 C-18 C-19
 T-20 T-21 S-5 S-6
 NEM-T-4

ILNTPELRKELVEVKGMADRIISMRTQLVSNLKKEGSSHNWQHITDQIG
 310 320 330 340 350
 CNBr-7 CNBr-8 CNBr-9 C-20 C-21 C-22 C-23 C-24
 T-22 T-23 T-24 S-7 S-8 S-9

MFCFTGLKPEQVERLTKEFSIYMTKDGRI SVAGVASSNVGYLAHAHQVTK
 360 370 380 390 400
 C-25 C-26 C-27 C-28 CNBr-10 C-29 C-30 C-30a T-25 T-26 T-27
 S-10
 NEM-T-5

DISCUSSION

All known amino acid sequences of AATases, i. e., the sequences of both the mitochondrial and cytosolic isoenzymes from pig (12-14) and chicken (present study, 16), the amino acid sequence of mAATase from rat (26), and partial sequences of mAATase from turkey (27) as well as AATase from E. coli (28) are compared in Fig. 2. The amino acid compositions of the different AATases are given in Table II. In order to obtain maximum agreement, deletions at both the N-terminus (positions 1 and 2) and the C-terminus (positions 411 and 412) and at positions 65, 127, 128, 131, 132, 153, and 407 have been introduced. The deletions at positions 127, 128, 131, and 132 differ from those assumed in the published amino acid sequence of mAATase from pig (11,13). As an additional criterion for arranging these deletions similarity regarding the polarity of the amino acid residues was considered. The polarities of the side chains were evaluated according to Ref. 29.

The following pages:

Fig. 2 - Comparison of the amino acid sequences of AATase isoenzymes from different species. The amino acid residues are numbered according to the sequence of cAATase from pig. Deletions are indicated by dashes. Lysine residue 258 which forms the aldimine bond with pyridoxal-5'-phosphate is shown in bold letter. A - Residues identical in mAATase and cAATase from all species are boxed in. B - Residues identical in the mitochondrial isoenzymes and residues identical in the cytosolic isoenzymes are boxed in. Hatching indicates "m-residues", i.e., residues that are invariant specifically in the mitochondrial isoenzymes (mAATase chicken = mAATase pig $\neq$ cAATase chicken $\neq$ cAATase pig, for details see text). In the comparison, the incompletely known sequence of the enzyme from E.coli is not considered.

¹ present study; ² from Barra et al., (27); ³ from Barra et al., (13); ⁴ from Huynh et al., (26); ⁵ from Shlyapnikov et al., (16); ⁶ from Doonan et al., (12); ⁷ from Kagamiyama et al., (28). The sequences of mAATase and cAATase from pig were also determined by Kagamiyama et al. (11), and by Ovchinnikov et al. (15), respectively. For the present comparison the sequences from Barra et al. (13) and Doonan et al. (12) were considered, because they contain more amide side chains.

A

1 mAAATase chicken
2 mAAATase turkey
3 mAAATase pig
4 mAAATase rat
5 mAAATase pig
6 CAATase chicken
7 CAATase pig
AATase E.coli

10 20 30 40 50

--S[S]W S H V E M G P P D P I L G V T E A F K R D T N S K K M N L G V G A Y R D D N G K P Y V L
--S[S]W A H V E M G P P D P I L G V T E A F K R D T N S K K M N L G V G A Y R D D N G K P Y V L
--S[S]W A H V E M G P P D P I L G V T E A F K R D T N S K K M N L G V G A Y R D D N G K P Y V L
--S[S]W T H V E M G P P D P I L G V T E A F K R D T N S K K M N L G V G A Y R D D N G K P Y V L
A C A S I I F A A V P R A P P I V A V F K L T A D I F R E D D G D S R K K V N L G V G A Y R T D E G Q P H V L
A P P S I V F A E V P Q A O P V L V F K L I A D F R E D D P D P R K K V N L G V G A Y R T D D C Q P H V L
M F Z B I T A A P A B P I L G L A B L F R A B Z P R G K I B L G I

mAAATase chicken
mAAATase turkey
mAAATase pig
mAAATase rat
CAATase chicken
CAATase pig

50 70 80 90 100

N C V R K I A E A M I A A K K - M D K E Y L P I A G L I A D F T R A S A E L A L G E N S E A F K S G R I Y
N C V R K I A E A M I A A K K - L D K E Y L P I I G G L I A E F F C K A S A E L A L G E N S E A F K S G R I Y
P S V R K I A E A Q I A A K N - L D K E Y L P I I G G L I A E F F C K A S A E L A L G E N S E A F K S G R I Y
P S V R K I A E A Q I A A K N - L D K E Y L P I I G G L I A E F F C K A S A E L A L G E N S E A F K S G R I Y
P V V R K I V E Q L I A G N S L N H E Y L P I I G L I A E F F R A N A S R I A L G D D S P A I A Q K R V
P V V R K I V E Q R I A N N S S L N H E Y L P I I G L I A E F F R T C A S R L A L G D D S P A L Q E K R V

mAAATase chicken
mAAATase turkey
mAAATase pig
mAAATase rat
CAATase chicken
CAATase pig

110 120 130 140 150

V T V Q I G I S G T G S L R V G A I N F L Q R F F K F S - - R D - - V Y L P K P S W G N H T P I F R D I A
V T V Q I G I S G T G S L R I I G A N F L Q R F F K F S - - R D - - V F L P K P S W G N H T P I F R D I A
V T V Q I G I S G T G A L R I I G A N F L Q R F F K F S - - R D - - V F L P K P S W G N H T P I F R D I A
V T V Q I G I S G T G A L R I I G A N F L Q R F F K F S - - R D - - V F L P K P S W G N H T P I F R D I A
G S V Q I G L G G T G A L R I I G A E F L L R W Y N G N N T D P V Y V S S P T W E N H N S V F M D I A
G G V Q I S L G G T G A L R I I G A E F L L R W Y N G N N T D P V Y V S S P T W E N H N G V F T T A

mAAATase chicken
mAAATase turkey
mAAATase pig
mAAATase rat
CAATase chicken
CAATase pig

160 170 180 190 200

G L - E L Q A Y R Y Y D I P K T C S L D F T G A M E D I S K I P E K S I I L H A C A H N P T G V D P
G M - Q L Q A Y R Y Y D I P K T C G F D F T G A L E D I S K I P A Q S V I L H A C A H N P T G V D P
G M - O L H S Y R Y Y D I P K T C G F D F T G A L E D I S K I P E Q S I F I L H A C A H N P T G V D P
G M - Q L E G Y R Y Y D I P K T C G F D F T G A L E D I S K I P E Q S I F I L H A C A H N P T G V D P
G F K D I R S Y R Y W D T E K R G L D L Q G F L S D L E N A P E F S I F V L H A C A H N P T G T D P
G F K D I R S Y R Y W D T E K R G L D L Q G F L S D L E N A P E F S I F V L H A C A H N P T G T D P

mAAATase chicken 210 R E E Q W K E L A I S V K K R N L L A Y F D M A Y Q G G F A S G D I N R D A W A I L R H F I E Q G I D V
 mAAATase turkey R P E Q W K E M A T L V K K N N L F A F F D M A Y Q G G F A S G D I N R D A W A V R H F I E Q G I N V
 mAAATase pig R P E Q W K E M A I A Y V K K N N L F A F F D M A Y Q G G F A S G D G N K D A W A V R H F I E Q G I N V
 mAAATase rat T P E Q W K Q I A A Y V M K R R C L F P F F D S A Y Q G G F A S G N L E K D A W A V R Y F V S E G F E L
 mAAATase chicken T P E Q W K Q I A I S V M K R R C L F P F F D S A Y Q G G F A S G N L E K D A W A I R Y F V S E G F E L
 mAAATase pig

mAAATase chicken 260 V L S I Q S Y A K N M G L Y G E R A G A F T V I C R D A E E A K R V E S Q L K I L I R P M Y S N P P M
 mAAATase turkey V L S I Q S Y A K N M G L Y G E R V G A F T V I C R D A E E A K R V Q S Q L K I L I R P M Y S N P P I
 mAAATase pig C L C Q S Y A K N M G L Y G E R V G A F T V V C K D A E E A K R V Q S Q L K I L I R P L Y S N P P L
 mAAATase rat C L C Q S Y A K N M G L Y N E R V G N L S V V G K D E D N V Q R V L S Q M E K I V R T T M S N P P S
 mAAATase chicken F C A Q S F S K N F G L Y N E R V G N L T V V A K E P D S I L R V L S Q M Q K I V R V T M S N P P A
 mAAATase pig F C A Q S F S K N F G L Y N E R V G N L T V V A K E P D S I L R V L S Q M Q K I V R V T M S N P P A
 AATase E.coli

mAAATase chicken 310 N G A R I I A S L I L N T P D L L R K E W L V E V K G M A D R I I S M R T Q L V S N L K K E G S S H N W
 mAAATase turkey N G A R I I A S L I L T P D L L R K E W L V E V K G M A D R I I S M R T Q L V S N L K K E G S S H N W
 mAAATase pig N G A R I I A S T I L T S P D L L R Q Q W L Q E V K G M A D R I I S M R T Q L V S N L K K E G S S H N W
 mAAATase rat Q G A R I I A T T L T S P Q L F A E W K D N V K T M A D R I L L M R S E L R S R L E S L G T P G T W
 mAAATase chicken Q G A R I I A T T L S D P E L F H E W T G N V K T M A D R I L L M R S E L R S R L E S L G T P G T W
 mAAATase pig

mAAATase chicken 360 Q H I T D Q I G M F C F T G L K P P E Q V E R L T K E F S I Y M T K D G R I S V A G V A S S N V G Y L
 mAAATase turkey Q H I T D Q I G M F C F T G L K P P E Q V E R L T K E F S I Y M T K D G R I S V A G V A S S N V G Y L
 mAAATase pig Q H I V D Q I G M F C F T G I K P P E Q V E R L T K E F S I Y M T K D G R I S V A G V T S G N V G Y L
 mAAATase rat N H I T D Q I G M F C F T G I K P P E Q V E R L T K E F S I Y M T K D G R I S V A G V T S G N V G Y L
 mAAATase chicken N H I T D Q I G M F S F T G L N P K I Q V E Y M I K E K H I Y L L M A S G R I N M C G L T T K N L D Y V
 mAAATase pig N H I T D Q I G M F S F T G L N P K I Q V E Y L I N Q K H I Y L L P S G R I N M C G L T T K N L D Y V

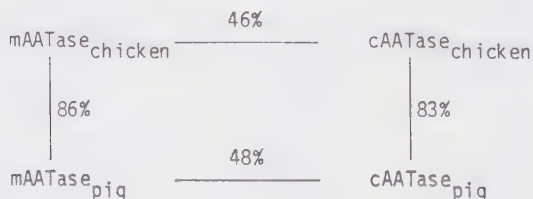
mAAATase chicken 410 A H A I H Q - V T K - -
 mAAATase turkey A H A I H Q - V T K - -
 mAAATase pig A H A I H Q - V T K - -
 mAAATase rat A H A I H Q - V T K - -
 mAAATase chicken A K S I H E A V T K I Q
 mAAATase pig A T S I H E A V T K I Q

TABLE II - Amino acid composition of different AATases

	mAATase			cAATase		AATase E.coli 8
	chicken 1	pig 2 3	rat 4	chicken 5	pig 6 7	
Lys	28	28	28	21	19	16.7
His	9	10	9	7	8	5.7
Arg	24	20	20	25	26	21.8
Asp	20	19 20	20	21	20 22	42.4
Asn	17	19 18	17	22	22 20	
Thr	17	19	19	23	25	22.3
Ser	29	25	26	27	26	18.1
Glu	25	20 21	22	22	23 25	47.6
Gln	13	19 18	18	15	18 16	
Pro	17	19	17	20	24	14.6
Gly	30	33	39	32	28	29.6
Ala	36	34	34	37	32	45.5
Cys	5	7	7	4	5	6.3
Val	24	27	26	29	29	25.4
Met	13	11	10	10	6	7.9
Ile	24	23	20	19	19	17.3
Leu	32	29	31	34	38	35.9
Tyr	15	13	13	12	12	11.3
Phe	16	19	19	21	23	19.9
Trp	7	7	6	9	9	4.4

¹ Present study; ² from Barra et al. (13); ³ from Kagamiyama et al. (11). Except the values for Asp, Asn, Glu, and Gln the amino acid composition is the same as given by Barra et al. in ²; ⁴ from Huynh et al. (26); ⁵ from Shlyapnikov et al. (16); ⁶ from Barra et al. (13); ⁷ from Ovchinnikov et al. (15). Except the values for Asp, Asn, Glu, and Gln the amino acid composition is the same as given by Barra et al. in ⁶; ⁸ from Kagamiyama et al. (28).

A substantial part (75%) of the primary structure of mAATase from turkey has been published by Barra et al.(27). As expected, there are only few, viz., 8 amino acid residues which differ from mAATase from chicken. The following Scheme is based on sequence information from Barra et al.(13), and Shlyapnikov (16) and gives the degree of sequence identity (in % of the total number of residues) of the isoenzymes from pig and chicken.



The two isoenzymes are homologous proteins as evidenced by the degree of their sequence identity close to 50% in both pig and chicken. The extent of sequence identity of the mitochondrial isoenzymes from chicken and pig is close to that reported for the pair of the cytosolic isoenzymes from the same species (16). The comparison of the primary structures confirms the conclusion drawn from

immunological data (17) that the degree of interspecies sequence identity both of the mitochondrial and of the cytosolic isoenzymes from chicken and pig markedly exceeds that of the intraspecies identity between mAATase and cAATase from chicken or from pig. These data indicate that the duplication of the common ancestor AATase gene occurred before the divergence of mammals and sauropsids. Assuming that mammals and sauropsids diverged about 300 million years ago (30) and that the mitochondrial and the cytosolic isoenzymes have developed with the same constant rate (see Ref 31), the gene duplication may be estimated to have occurred about 1000 million years ago, i.e., at the time of the emergence of eucaryotic cells (32).

The amino acid sequence of the pyridoxal-phosphate binding tetrapeptide and the 33 N-terminal residues of the single AATase from E. coli have been reported (28). This partial sequence indicates that the enzyme from E. coli is homologous to the vertebrate isoenzymes and shows a similar degree of relatedness to the sequence of the mitochondrial and cytosolic isoenzyme. Out of 33 N-terminal residues 12 and 11 are identical with the vertebrate mitochondrial and cytosolic enzyme, respectively.

There are 166 residues (40% of total) which are invariant in all four enzymes (see also Ref. 33), the greater portion of them (63%) are apolar amino acids (Fig. 2 A). For comparison, of the total 401 amino acid residues only 55% are apolar. (The polarities were evaluated according to Ref. 29). As expected and established by the determination of the three-dimensional structure (8) the majority of these invariant residues are located in the interior of the molecule, in the active site cleft, or at the subunit interface. Apparently, these residues contribute to the formation of functionally important structures common to both isoenzymes.

In both the mitochondrial and cytosolic AATase from chicken, the sum of arginine plus lysine residues exceeds that of glutamic acid plus aspartic acid residues. The positive net charge is higher in the mitochondrial isoenzyme, consistent with its higher isoelectric point (Table III). Similar ratios between basic and acid residues have been reported for the isoenzymes from pig (11,13).

TABLE III - Content of charged amino acid residues and isoelectric points of AATase isoenzymes from pig and chicken

	Lys + Arg	Asp + Glu	Charge difference (basic minus acid residues) ^a	pI
mAATase _{chicken}	52	45	7 +	9.3 ^e
mAATase _{pig}	48	39 ^b	9 +	9.0 ^f
cAATase _{chicken}	46	43 ^c	3 +	6.5
cAATase _{pig}	45	43 ^d	2 +	5.7 ^g

^aIonizable residues other than Lys, Arg, Glu, and Asp are not considered.

^bFrom Ref (13), ^cfrom Ref (16), ^dfrom Ref (13), ^eKirsten, H., and Sandmeier, E., unpublished data, ^ffrom Ref (34), ^gfrom Ref (3, 35)

The following comparison of the primary structures of the two isoenzymes both from pig and chicken attempts to identify structural features which might relate to functional features specific for the mitochondrial isoenzyme, e.g., its specific posttranslational uptake into the mitochondria (see Introduction). The selection criterion for identifying such residues was identity in the two mitochondrial AATases coincident with nonidentity between them and either one of the cytosolic isoenzymes as well as nonidentity between the two cytosolic AATases themselves (mAATase chicken = mAATase pig $\neq$ cAATase chicken $\neq$ cAATase pig). The criterion of nonidentity excludes any residues that are essential for structural or functional features common to both cytosolic and mitochondrial AATase. In the total sequence there are 36 residues (9% of total) that comply with the above selection criterion; henceforth, we denote them as "m-residues" because they are conserved specifically in the mitochondrial AATase. If not only identity but also conservative substitutions of amino acid residues are taken into consideration only one additional amino acid residue (position 81) complies with the definition of "m-residues". For comparison, there are only 10 residues (2.4% of total) which are identical specifically in the two cytosolic isoenzymes (cAATase chicken = cAATase pig $\neq$ mAATase chicken $\neq$ mAATase pig). Including mAATase from rat which shows 94% sequence identity with mAATase from pig into this comparison, two "m-residues" (position 62 and 145) are not conserved and thus may not be considered essential specifically for the mitochondrial isoenzyme. The identification of "m-residues" according to the criteria given above requires that the amino acid sequence not only of the mitochondrial but also of the cytosolic isoenzyme of the same species is known and is considered in the comparison. The greater the evolutionary distance between two species the more significant is their comparison with respect to the identification of "m-residues". Many

TABLE IV - Charged amino acids among the "m-residues" and at the corresponding positions in the cytosolic isoenzymes

	Lys + Arg	Glu + Asp	Net charge ^a	Charge difference (mitochondrial minus cytosolic)	Charge difference in the total protein
Chicken					
mAATase	8	1	7+	7+	4+
cAATase	3	3	0		
Pig					
mAATase	8	1	7+	10+	7+
cAATase	1	4	3-		

^aIonizable residues other than Lys, Arg, Glu, and Asp are not considered

"m-residues" identified by comparing evolutionarily distant species, e. g., chicken and pig, will not comply with the same selection criterion if more closely related species are compared, because in many positions of "m-residues" the two cytosolic isoenzymes will not differ. For this reason mAATase from rat and turkey have not been included into the present comparison.

There is a remarkable difference in charge between the "m-residues" and the amino acid residues in the corresponding positions in the cytosolic enzymes (Table IV). Among the 36 "m-residues", the number of basic amino acids markedly exceeds that of acidic amino acids. In both species the charge difference between the mitochondrial and the cytosolic isoenzymes in the positions of the "m-residues" exceeds the overall charge difference between the two isoenzymes. Among the "m-residues" the share of polar amino acid residues (57%) is larger than in the total protein (45%).

The "m-residues" appear to cluster in certain segments of the polypeptide chain. (In Fig. 2 B the "m-residues" are hatched). These segments are conspicuous by their evolutionary conservatism specifically in the mitochondrial isoenzyme. Table V lists the segments of the polypeptide chain which comply with the two following criteria: (1) The degree of interspecies identity between the two mitochondrial enzymes (mAATase chicken = mAATase pig) exceeds the average degree of identity of the two polypeptide chains. The length of the segment is taken such that the degree of identity is maximum. (2) In contrast, the degree of interspecies identity between the two cytosolic AATases (cAATase chicken = cAATase pig) is lower than the average of the polypeptide chain. In all segments fulfilling these two criteria ("m-segments"), the degree of intraspecies identity between the cytosolic and mitochondrial AATases (cAATase chicken = mAATase chicken, and cAATase pig = mAATase pig) proved to be markedly below average. The "m-segments" comprise 151 amino acid residues (38% of the total polypeptide

TABLE V - Segments of the polypeptide chain with an above average interspecies sequence identity between the mitochondrial isoenzymes and a below average interspecies identity between the cytosolic isoenzymes ("m-segments")

Abbreviations used in this Table: m_c , mAAATase_{chicken}, m_p , mAAATase_{pig}, c_c , cAAATase_{chicken}, c_p , cAAATase_{pig}

Position	Degree of sequence identity, %				Number of "m-residues" in segment
	$m_c = m_p$	$c_c = c_p$	$c_c = m_c$	$c_p = m_p$	
30 N-terminal residues	97	63	27	30	7
82- 86	100	60	0	20	1
96-102	100	57	14	14	3
120-149	97	77	27	23	5
161-166	100	67	17	17	2
170-180	91	73	27	27	2
269-295	93	70	27	33	7
330-351	100	77	32	32	1
372-384	100	69	31	31	2
Total polypeptide chain (1-412)	86	83	46	48	36

chain) and contain not only 30 out of the total 36 "m-residues", but also 6 out of the 10 and 11 deletions in the mitochondrial isoenzymes from chicken and pig, respectively.

There are only two segments which comply with corresponding criteria for the cytosolic isoenzymes (152-156 and 249-253) each comprising 3 "c-residues" among 5 amino acid residues in total.

In the spatial structure of the mitochondrial enzyme from chicken (8) nearly all "m-residues" are found to be located on the surface of the molecule (Fig. 3 A). The greater part of the "m-segments" listed in Table V build up an apparently contiguous surface region located in the lower part of the molecule (Fig. 3 B). The existence of a surface region behaving conservatively during evolution specifically in the mitochondrial isoenzyme implies additional evolutionary constraints imposed on its structure. These constraints might relate to the transport of the mitochondrial isoenzyme from its site of synthesis in the cytosol through the mitochondrial double membrane into the mitochondrial matrix. Both in a cell-free protein synthesizing system directed with total m-RNA from chicken heart (1) and after *in vitro* read-out translation of free polysomes from chicken liver (2) as well as in cultured chicken embryo fibroblasts (36) mAAATase was found to be synthesized as a precursor molecule with higher molecular weight. Apparently, it is translocated into the mitochondria after completion of translation (2, 36). A hypothetical mechanism of translocation may involve uptake as a monomer with the extrapeptide preventing dimerization. The present data suggest that not only the prepeptide but also additional surface regions might partici-

pate in the specific interaction with the mitochondrial double membrane. However, it cannot be excluded that the additional evolutionary constraints imposed on the structure of the mitochondrial isoenzyme might relate to other specific features, e.g., to an independent regulation of its degradation or to interactions with other mitochondrial proteins (37).



Fig. 3 - "m-Residues" and "m-segments" in the spatial structure of mAATase. A wire model of one subunit of mAATase from chicken is shown. The model is based on data from X-ray crystallographic studies (8). A - The "m-residues" are symbolized by black balls. C-Terminus and lysine 258 are marked by circles. B - "m-Segments" are marked by dotted lines. N-Terminus, C-terminus, and lysine 258 are marked by black balls.

It is tempting to assume that all PLP-dependent enzymes constitute one great superfamily or a few families of proteins, i.e., originate from a single or a few ancestor PLP protein(s). Stereochemical studies, viz. the generality of protonation from the exposed si face at C-4' of PLP-substrate imines and the similarity of conformational changes upon substrate binding, document the relatedness of these proteins (38). Furthermore, in all PLP-dependent enzymes the coenzyme is bound to the ϵ -aminogroup of a lysine residue by a Schiff base. In most of the known PLP binding sites the partial amino acid sequence Ser-X-X-Lys(Pxy) is a common structural feature (39).

Aspartate aminotransferase is the first and to date the only PLP-dependent enzyme, of which the covalent structure and the spatial structure at atomic resolution have been determined. The elucidation of the primary and spatial structure of other PLP-dependent enzymes will eventually not only allow to compare their structures thus gaining insight into their evolutionary relationship but might also reveal the molecular basis of the diverse reaction specificities of the many different PLP-dependent enzymes.

SUPPLEMENTARY MATERIAL

TO

THE COVALENT STRUCTURE OF MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE FROM CHICKEN. IDENTIFICATION OF SEGMENTS OF THE POLYPEPTIDE CHAIN INVARIANT SPECIFICALLY IN THE MITOCHONDRIAL ISOENZYME

1. EXPERIMENTAL PROCEDURE

Materials - Mitochondrial aspartate aminotransferase (mAATase)¹ was reduced with dithiothreitol in 6 M guanidine hydrochloride, 0.1 M Tris chloride (pH 8.0) for 3 h and carboxymethylated with iodo-2-[¹⁴C] acetic acid (40) or aminoethylated (41). Guanidine hydrochloride, sequanal grade, and sodium iodoacetate were from Pierce, cyanogen bromide from Fluka, iodo-2-[¹⁴C] acetic acid from Amersham, dithiothreitol from Calbiochem. The reagents for Edman degradation were sequence grade from Pierce or Fluka. Carboxypeptidase A (CPA)¹, carboxypeptidase B (CPB)¹ (treated with phenylmethylsulfonyl-fluoride), and trypsin (treated with L-1-tosylamido-2-phenylethyl-chloromethylketone) were purchased from Worthington; carboxypeptidase Y (CPY)¹ and α-chymotrypsin were from Boehringer, aminopeptidase M (APM)¹ from Roehm/Roth, and Staphylococcus aureus protease V8 was from Miles. Aeromonas aminopeptidase (AAP)¹ was prepared as described elsewhere (42). Cellulose chromatographic sheets were from Eastman.

¹ The abbreviations used are:
mAATase, mitochondrial aspartate aminotransferase,
CPA, carboxypeptidase A,
CPB, carboxypeptidase B,
CPY, carboxypeptidase Y,
APM, aminopeptidase M,
AAP, Aeromonas aminopeptidase,
DMBA, dimethylbenzylamine,
DABITC, 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate,
PTH, phenylthiohydantoin,
DABTH, 4-N, N-dimethylaminoazobenzene 4'-isothiohydantoin,
HPLC, high performance liquid chromatography,
PITC, phenylisothiocyanate,
NEM, N-2,3-[¹⁴C]ethylmaleimide.
NMM, N-methylmorpholinium trifluoroacetate

Thin-layer chromatography and high voltage electrophoresis - Small peptides were purified by thin-layer chromatography on cellulose (1-butanol / acetic acid / water / pyridine, 13 / 3 / 12 / 10). Preparative and analytical high-voltage electrophoresis was performed on Whatman 3 MM paper at pH 1.9 (acetic acid / formic acid / water, 4 / 1 / 45), at pH 3.5 (pyridine / acetic acid / water, 1 / 10 / 89), and at pH 6.5 (pyridine / acetic acid / water, 25 / 1 / 225). Both thin-layer chromatograms and high-voltage electropherograms were stained with fluorescamine (Roche) in acetone and eluted with 50% acetic acid.

Determination of amino acid composition and sequence analysis - After acid hydrolysis under vacuum (6 N HCl, 110⁰, 22 h), the amino acids were analyzed on a Durrum D-500 analyzer using the standard sodium citrate buffers for protein hydrolysates. Automatic Edman degradation was performed with a liquid-phase Beckman Sequencer (model 890 B, updated) using either the dimethylbenzylamine buffer system (DMBA)¹ or the quadrol buffer system and the Beckman peptide program 060275. 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC)¹ was used as indicated (21). Some peptides were modified with 2-amino-1,5-naphthalene disulfonic acid by the method of Foster (43). In some runs, 3 mg polybrene (EGA-Chemie) was added (44, 45) which had been purified by chromatography on Dowex-1 (G. Frank, personal communication). Two peptides were sequenced by the automated procedure on a LKB 4020 solid phase sequencer as described earlier (46, 47).

Phenylthiohydantoin (PTH)¹ and 4-N,N-dimethylaminoazobenzene 4'-thiohydantoin (DABTH)¹ amino acid derivatives were identified by gas liquid chromatography (48, 49) or by high performance liquid chromatography (HPLC)¹ (Waters Associates, model ALC/GPC-204) according to the procedure of Frank and Strubert (50) or Wilson et al. (20), respectively. PTH or DABTH derivatives of [¹⁴C] carboxymethylated cysteine were identified by their radioactivity. In addition, amino acid analyses were performed after acid hydrolysis (6 N HCl containing 0.1% stannous chloride at 150⁰C for 4 h in the case of PTH-derivatives (51) and 12 h in the case of DABTH-derivatives (20)). Manual Edman degradation of smaller peptides was performed with phenylisothiocyanate (PITC)¹ (52) or with DABITC (53).

2. CYANOGEN BROMIDE PEPTIDES

For cyanogen bromide cleavage, the procedure by Gross (54) in 70% formic acid was followed. Cyanogen bromide peptides were separated by gel filtration (Fig. 4) and pooled as indicated.

Fraction I contained the largest cyanogen bromide peptide, CNBr-5, which was used directly for automated sequence analysis (Table VI). The amino acid compositions of this and the other 9 cyanogen bromide peptides that were isolated are given in Table VII.

Fraction II and III were purified by HPLC on a reverse phase column (55). Chromatography of fraction II gave 3 main peaks (Fig. 5), one of them contained a single peptide, CNBr-10, which was sequenced by the automated procedure (Table VI). According to its amino acid composition fraction II-2 contained cyanogen bromide peptide 167-215, which was not further analyzed. Cyanogen bromide fraction III gave two pure peptides (Fig. 6): CNBr-7, which was sequenced up to residue 7 (Table VI), and CNBr-2, for which only the amino acid composition was determined (Table VII). Fraction III-1 contained no amino acid residues. Fraction III-2 was rechromatographed (Fig. 7) and yielded CNBr-3 and CNBr-9. The sequence of CNBr-3 is given in Table VI. The amino acid compositions of the peptides from a Staphylococcus aureus protease V8 digestion of CNBr-9 (Table VIII) identified its position in the sequence. The C-terminus was determined by CPY- and CPA-digestion (Table IX). Cyanogen bromide fraction IV was chromatographed on Sephadex G-25 (Fig. 8). Fraction IV-2 and IV-3 were subjected to thin-layer chromatography on cellulose. Fraction IV-2 contained two peptides, CNBr-4 and CNBr-8. The sequences of both peptides were determined by digestion with exopeptidases (Table IX) and by tryptic digestion with separation of the ensuing peptides with high voltage electrophoresis at pH 1.9 (Table VIII). Fraction IV-3 contained CNBr-6 which was sequenced by the automated procedure (Table VI). The results of exopeptidase digestion of CNBr-6 are given in Table IX. Fraction IV-5 contained the single N-terminal peptide CNBr-1. After desalting on a Biogel column (200-400 mesh, 0.9 x 130 cm) in 0.1 M ammonium bicarbonate its N-terminus was determined by exopeptidase digestion (Table IX). In fraction IV-1 and IV-4 the peptide concentration was too low for further analysis.

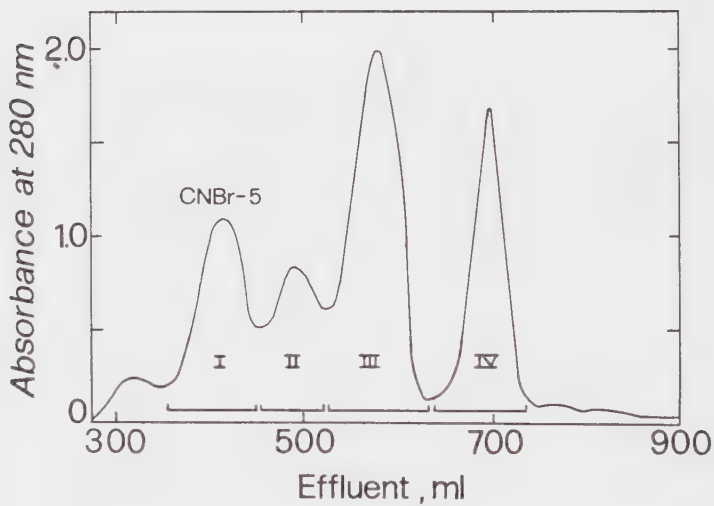


Fig. 4 - Chromatography of cyanogen bromide peptides from S-carboxymethylated mAATase on Sephadex G-75 (2.6 x 140 cm-column in 10% aqueous formic acid). The bars indicate fractions pooled.

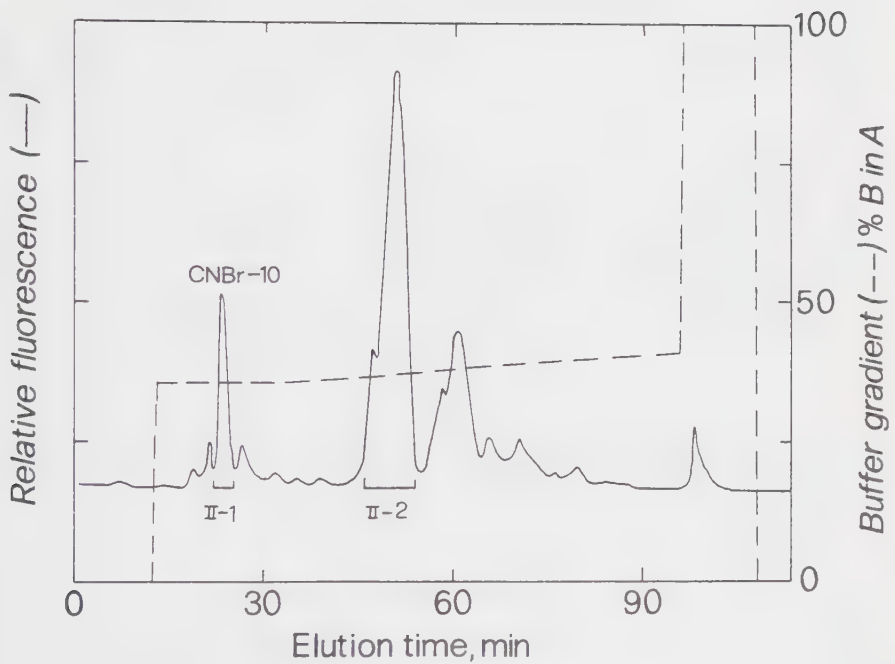


Fig. 5 - HPLC separation of cyanogen bromide peptides of fraction II (Fig.4). Chromatography was on Lichrosorb RP 18 (10 μ m particle size, 4.6 x 250 mm-column) developed with a gradient (dashed line) at 0.7 ml/min using as buffer A, 0.125 M pyridine formate, pH 3.0, and as buffer B, 1.0 M pyridine acetate, pH 5.5, 60% (v/v) 1-propanol. Part of the eluate (3%) was analyzed with fluorescamine.

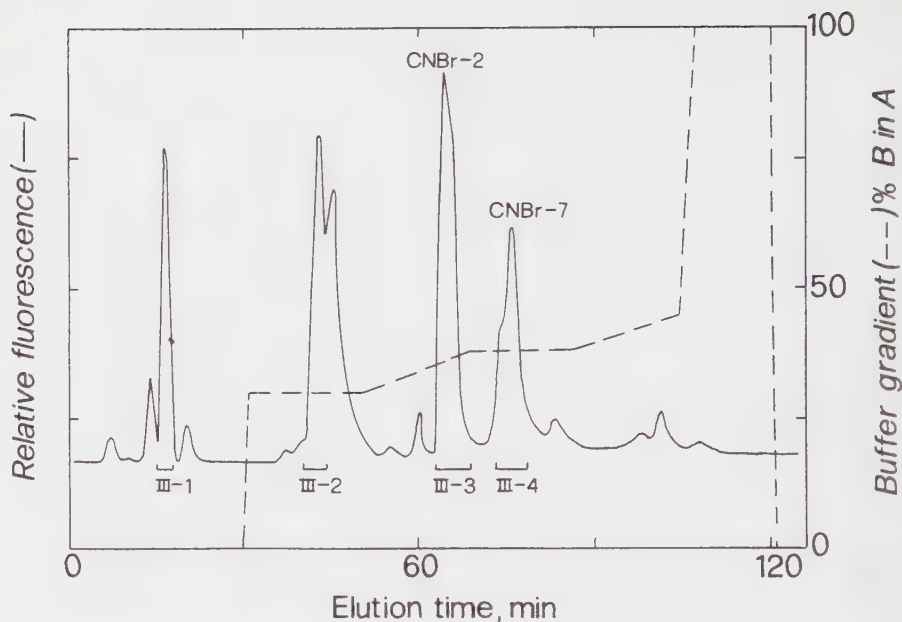


Fig. 6 - HPLC separation of cyanogen bromide peptides of fraction III (Fig.4). The chromatography was carried out as in Fig 5.

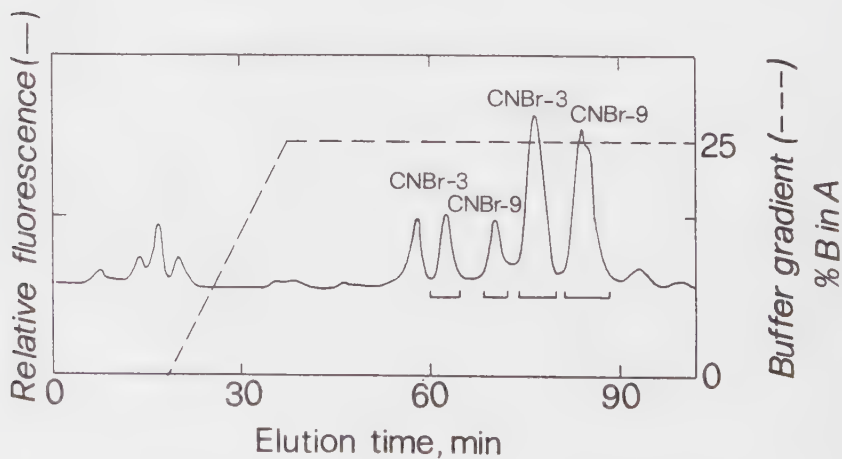


Fig. 7 - Re-chromatography by HPLC of fraction III-2 of Fig. 6. Analysis with fluorescamine was performed with 2% of the eluted material. Separation of each CNBr-3 and CNBr-9 into two fractions is due to their different C-terminal residues, homoserine and homoserine lactone.

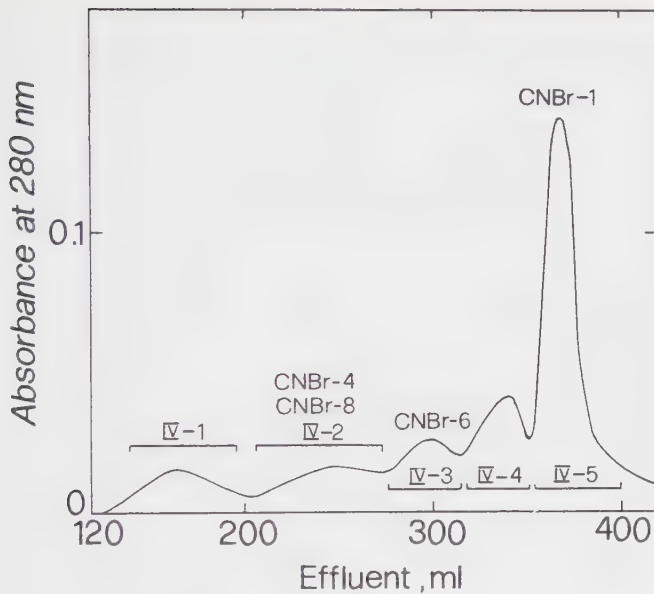


Fig. 8 - Chromatography of cyanogen bromide peptides of fraction IV (Fig. 4) on Sephadex G-25 (1.9 x 150 cm-column) in 1 M acetic acid.

TABLE VI - Edman degradation of cyanogen bromide peptides by automated liquid phase sequencer

Peptide	Amount nmol	Edman reagent	Solvent	Results ^a
CNBr-3	14	DABITC	Quadrol + Polybrene	N L G V G A Y R D D N G K P Y V L N C V R K A ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓
CNBr-5	250	PITC	Quadrol	D K E Y L P I A G L A D F T R A S A E L A L G E N S E A F K S G R Y V T V Q G I S G T G S L R V G A N ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓
CNBr-6	250	PITC	DMBA	Y S N P P ↓ ↓ ↓ ↓ ↓
CNBr-7	10	DABITC	Quadrol + Polybrene	N G A R I A S ↓ ↓ ↓ ↓ ↓
CNBr-10	20	DABITC	Quadrol + Polybrene	T K D G R I S V A G V A S S N V G Y L ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓

^a ↓ Identification by HPLC
 ↓ Identification by amino acid analysis on Durrum analyzer
 ↓ Identification by thin layer chromatography on silica gel
 The repetitive yields were 92 to 97%

TABLE VII - Amino acid compositions of S-carboxymethylated cyanogen bromide peptides

	CNBr-1	CNBr-2	CNBr-3	CNBr-4	CNBr-5	CNBr-6	CNBr-7	CNBr-8	CNBr-9	CNBr-10
Lys	1.1(1)	3.1(3)	2.0(2)	2.0(2)	5.5 (5) 1.5 (1)		1.7(2)		1.7(2) 1.6(2)	2.0(2) 1.9(2)
His									1.0(1)	0.9(1)
Arg		1.1(1)	2.1(2)		6.6 (7)		1.6(2)	0.9(1)	2.6(3)	2.1(2)
Asp		3.1(3)	4.9(5)		8.1 (9)	1.1(1)	1.9(2)	1.0(1)		
Thr		1.8(2)			5.0 (6)		0.9(1)		1.4(2)	1.8(2)
Ser	2.6(3)	0.9(1)			6.4 (8)	1.0(1)	1.1(1)	1.0(1)	2.0(3)	2.6(3)
Glu	1.0(1)	1.1(1)	1.1(1)		8.7 (8)		2.8(3)		3.4(4)	1.0(1)
Pro		2.8(3)	0.9(1)		4.5 (5)	1.8(2)	1.0(1)			
Gly		2.1(2)	3.0(3)		9.7(10)		1.9(2)		1.8(2)	3.0(3)
Ala		1.2(1)	2.9(3)	2.0(2)	9.8(10)		1.8(2)	1.0(1)		4.0(4)
Cys			0.7(1)		0.3 (1)					
Val	1.0(1)	0.8(1)	2.9(3)		5.1 (4)		1.6(2)		1.1(1)	3.6(4)
Met ^a	1.1(1)	0.7(1)	0.7(1)	1.0(1)	0.5 (1)	0.9(1)	0.9(1)	0.8(1)	0.8(1)	
Ile		1.0(1)		1.0(1)	3.8 (3)		1.6(2)	1.5(2)	1.5(2)	1.8(2)
Leu		0.6(1)	2.0(2)		10.0(10)		3.2(4)		1.7(2)	1.0(1)
Tyr			1.9(2)		4.2 (6)	1.0(1)				0.7(1)
Phe		0.8(1)			7.0 (8)					
Trp	(2)				(1)		(1)		(1)	
Total										
residues	9	22	26	6	103	6	26	7	26	28
% Yield	37	69	55	43	77	60	45	65	39	15
Charge ^b					0/+					
Position	1-9	10-31	32-57	58-63	64-166	287-292	293-318	319-325	326-351	374-401

^a Homoserine + homoserine lactone^b As derived from electrophoretic mobility at pH 6.5. Due to the inhomogeneous C-terminus two peptides differing in charge were found.

TABLE VIII - Digestion of cyanogen bromide peptides

Digestions with trypsin and *Staphylococcus aureus* protease were carried out at 37°C in 0.1 M ammonium bicarbonate, pH 8.5 and 7.8, for 2 h and 18 h, respectively. Peptides were separated by high voltage electrophoresis at pH 1.9 or 6.5

	Tryptic peptides from		Peptides obtained by digestion with			
	CNBr-4		CNBr-8		<i>Staphylococcus aureus</i> protease V8 of CNBr-9	
	T-1 ^a	T-2 ^b	T-1 ^c	T-2 ^d	S-1 ^e	S-2 ^f
Lys	1.1(1)	1.0(1)			2.0(2)	
His						1.8(2)
Arg			1.0(1)		1.0(1)	
Asp			1.0(1)		1.1(1)	2.0(2)
Thr					0.7(1)	0.9(1)
Ser				1.0(1)	0.8(1)	1.3(2)
Glu					1.8(2)	1.2(1)
Gly						0.6(1)
Ala	2.0(2)		1.0(1)			
Val					0.7(1)	
Met		0.8(1)		1.0(1)		
Ile	1.0(1)			2.1(2)		0.8(1)
Leu					1.7(2)	
Trp						(1)
Charge ^b	0					

The amino acid compositions correspond to the following segments (Fig. 1):

^a58-61; ^b62-63; ^c319-321; ^d322-325; ^e326-336; ^f337-347

^bAs derived from electrophoretic mobility at pH 6.5

TABLE IX - Exopeptidase digestion of cyanogen bromide peptides

C- and N-Terminal sequences of isolated peptides were determined using AAP in 0.02 M Tricine chloride, pH 8.0, CPY in 0.1 M pyridine acetate, pH 5.5, CPA and CPB in 0.2 M N-ethylmorpholine acetate, pH 8.5 at 37°C

Peptide	Amount nmol	Enzyme	Molar ratio	Time min	Residues released mol/mol
CNBr-1	8	AAP	1:250	45	Ser 0.9
CNBr-4	6	AAP	1:1000	15	Ile 0.8, Ala 0.6
	30	CPA/B	1:100	45, 45	Homoser 1.0, Lys 1.0, Ala 0.4
CNBr-6	8	AAP	1:100	60	Tyr 0.6
	8	APM	1:100	45	Tyr 0.4, (Ser, Asn) 0.5
CNBr-8	8	APM	1:100	60	Ala 1.0
	8	CPA	1:1000	45	Homoser 0.5, Ser 0.2
CNBr-8-T-2	7.4	AAP	1:1000	15	Ile 1.5
CNBr-9	6	CPY	1:17	20	Homoser 0.2, Gly 0.2, Ile 0.1
	17	CPA	1:5	120	Homoser 0.6, Gly 0.5, Ile 0.4, Glu 0.2

3. CHYMOTRYPTIC PEPTIDES

The cyanogen bromide peptides of fractions II and III in Fig. 4 were digested with chymotrypsin (3% by weight, 3 h at 37°C in 0.1 M ammonium bicarbonate, pH 8.5). The ensuing peptides were separated by ion-exchange chromatography (Fig. 9 and 10). Pooled fractions were desalted on Sephadex G-25 in 0.1 M ammonium bicarbonate. The bars in Fig. 9 and 10 indicate fractions containing a peptide which could be sequenced without further purification. The other peptides were purified by preparative high voltage electrophoresis and thin-layer chromatography. Some peptides were subjected to total hydrolysis by leucine aminopeptidase (Worthington) (56) in order to determine whether they contained Asp (Glu) or Asn (Gln). The amino acid compositions of all chymotryptic peptides are summarized in Tables X and XI. The results of their Edman degradation are given in Table XII, and those from digestions with exo- and endopeptidases in Tables XIII and XIV, respectively.

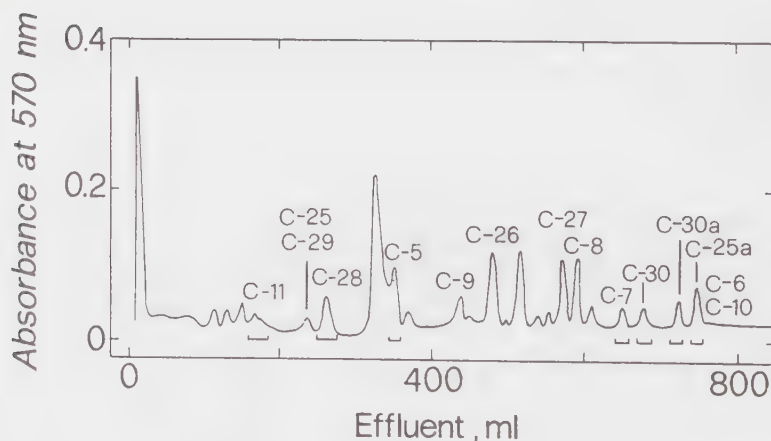


Fig. 9 - Chromatography of chymotryptic peptides from cyanogen bromide fraction II (Fig. 4) on a column (0.9 x 20 cm) of M-72 cation exchange resin (Beckman). Following equilibration with 0.05 M pyridine acetate, pH 2.5, at 55°C, the peptides were eluted with a four-chamber gradient containing 200 ml of 0.05 M pyridine acetate, pH 2.5, 200 ml of 0.2 M pyridine acetate, pH 3.1, 200 ml of 0.5 M pyridine acetate, pH 3.75, and 200 ml 2.0 M pyridine acetate, pH 5.0. The eluate was monitored by ninhydrin reaction after alkaline hydrolysis.

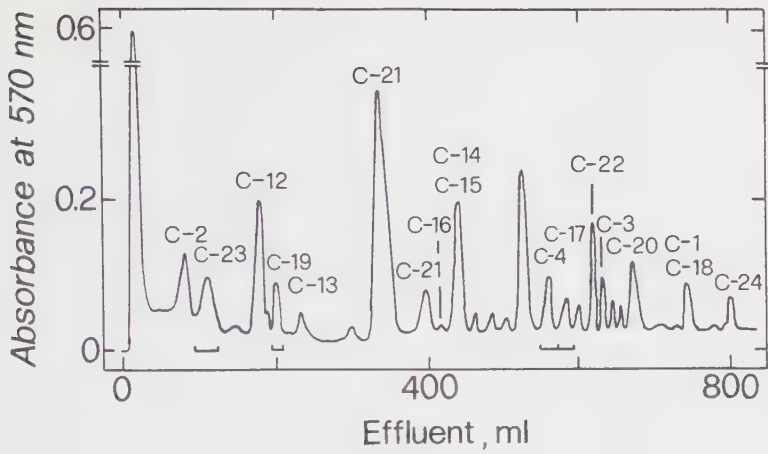


Fig. 10 - Chromatography of chymotryptic peptides from cyanogen bromide fraction III (Fig. 4) on a M-72 cation exchange resin as described in Fig. 9.

TABLE X - Amino acid compositions of chymotryptic peptides obtained by digestion of cyanogen bromide fraction II (Fig.4)
The chromatographic separation of these peptides is shown in Fig. 9

	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-25	C-25a	C-26	C-27	C-28	C-29	C-30	C-30a
Lys	1.8(2)	1.0(1)		1.0(1)	1.0(1)					0.8(1)	1.0(1)				
His			1.1(1)			1.0(1)				1.0(1)				1.8(2)	2.0(2)
Arg	1.2(1)					1.2(1)	1.0(1)								
Asp										0.6(1)	0.9(1)				
Thr															
Ser	1.7(2)		2.8(3)	1.1(1)	0.9(1)					2.7(3)	1.0(1)	0.8(1)			
Glu	1.9(2)		1.0(1)							0.9(1)					
Pro	1.0(1)									1.1(1)					
Gly													1.1(1)		
Ala		2.0(2)			0.9(1)		0.9(1)							1.9(2)	2.0(2)
Cys		1.0(1)			2.0(2)			0.6(1)	1.0(1)	1.0(1)			1.0(1)		
Val ^a							0.9(1)								
Ile	3.2(4)											1.0(1)		1.0(1)	
Leu	1.2(1)			1.0(1)		2.1(2)				2.0(2)				1.1(1)	
Tyr							1.0(1)					0.9(1)	0.9(1)		
Phe							1.2(1)	2.0(2)	1.1(1)		0.9(1)				
Trp			(1)												
Total residues	13	4	6	3	5	4	5	3	2	11	4	3	3	6	5
% Yield ^b	86	32	16	38	52	15	27	45	33	23	87	80	18	27	28
Charge ^b				0	0		0/-			0	0				
Position	167-179	182-185	192-197	198-200	201-205	207-210	211-215	352-354	353-354	355-365	366-369	370-372	389-391	392-397	393-397

^a Homoserine + homoserine lactone

^b As derived from electrophoretic mobility at pH 6.5. In the case of C-11 two peptides differing in charge were found due to the inhomogeneous C-terminus.

Table XI - Amino acid compositions of chymotryptic peptides obtained by digestion of cyanogen bromide fraction III (Fig. 4)
The chromatographic separation of these peptides is shown in Fig. 10

	C-1	C-2	C-3	C-4	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20	C-21	C-22	C-23	C-24
Lys	0.9(1)		1.0(1)	0.8(1)					0.5(1)	1.0(1)			1.0(1)	0.9(1)			1.7(2)
His	1.1(1)		0.9(1)						2.0(2)				1.0(1)				0.9(1)
Arg	2.0(2)		3.1(3)				1.0(1)		1.2(1)								
Asp	0.9(1)	0.8(1)							0.5(1)							0.9(1)	0.9(1)
Thr									1.0(1)								
Ser									4.2(4)			1.0(1)					
Glu				1.0(1)	1.6(2)		1.0(1)										
Pro		2.2(2)	1.0(1)		1.1(1)				2.0(2)				2.2(2)	1.2(1)			1.6(2)
Ala		1.1(1)	1.2(1)	2.0(2)		1.0(1)	0.8(1)	1.0(1)	0.8(1)				0.7(1)				1.0(1)
Val							1.1(1)	1.0(1)	1.9(2)								
Met ^a		1.0(1)		0.7(1)													1.0(1)
Ile										1.0(1)							
Leu		1.1(1)				0.9(1)			0.7(1)	1.0(1)			0.9(1)	0.9(1)	1.0(1)		
Tyr		1.0(1)				1.0(1)			0.9(1)	1.0(1)							
Phe								0.9(1)									
Trp																	
Total residues	5	7	8	5	4	3	4	3	17	3	4	2	7	5	4	4	7
Yield	13	13	48	60	60	71	75	68	39	45	27	29	22	8	26	65	5
Chromatographic position	23-27	32-38	39-46	53-57	245-248	253-255	256-259	260-262	263-279	280-282	283-286	299-300	305-311	312-316	326-329	330-333	334-340

^ahomoserine homoserine lactone

^bAs derived from electrophoretic mobility at pH 6.5. In the case of C-4 two peptides differing in charge were found due to the inhomogeneous C-terminus.

TABLE XII - Edman degradation of chymotryptic peptides

Peptide	Amount nmol	Edman reagent	Solvent	Edman degradation	Results ^a
C-1	90	PITC	pyridine	manual	K R
C-2	10	DABITC	pyridine	manual	N L G
C-3	50	PITC	pyridine	manual	R D D N
C-4	100	PITC	pyridine	manual	K A E A
C-5	400	PITC	DMBA	automated liquid phase	E D I S K I P E K S I
C-6	8	PITC	pyridine	manual	A C A
C-7	250	PITC	DMBA	automated liquid phase	P
C-11	18	DABITC	pyridine	manual	A Y F D
C-12	10	PITC	pyridine	manual	S Q S Y
C-13	50	PITC	pyridine	manual	G L Y
C-14	12	DABITC	pyridine	manual	G E R
C-16	16	DABITC	pyridine	manual	T V I C
C-16-T-3	9	DABITC	pyridine	manual	V E S ^{cpm}
C-17	60	PITC	pyridine	manual	K
C-18	50	PITC	pyridine	manual	I R P
C-19	20	DABITC	pyridine	manual	S L
C-20	35	DABITC	pyridine	manual	P E L R
C-21	15	DABITC	pyridine	manual	L V
C-22	30	PITC	pyridine	manual	R T Q L
C-23	200	PITC	pyridine	manual	V S N L
C-24	29	PITC	pyridine	manual	K K E G S
C-25	60	PITC	pyridine	manual	F
C-26	240	PITC	DMBA	automated liquid phase	T G L K P E Q V E
C-29	40	PITC	pyridine	manual	V G Y

^a $\rightarrow$ Identification by HPLC
 $\rightarrow$ Identification by amino acid analysis on Durrum analyzer
 $\rightarrow$ Identification by thin-layer chromatography on silica gel
 $\rightarrow$ Identification by gas liquid chromatography
 $\rightarrow$ Identification by difference analysis
 $\rightarrow$ ¹⁴C Carboxymethylated cysteine identified by its radioactivity
 cpm The repetitive yields were 91 to 96%

TABLE XIII - Exopeptidase digestion of chymotryptic peptides

In addition to the exopeptidases used for cyanogen bromide peptides (Table IX) in one case leucine aminopeptidase (LAP) was used for total hydrolysis in 5 mM Tris chloride, pH 8.5, 5 mM magnesium chloride, 40°C, 24 h

Peptide	Amount nmol	Enzyme	Molar ratio	Time min	Residues released mol/mol
C-3	6 4	APM CPA	1:50 1:100	45 20	Arg 0.5 Tyr 1.0
C-4	6 4	CPA LAP	1:100 1:55	45 1440	Homoser 0.6 Lys 1.0, Ala 1.5, Glu 1.0, Homoser 0.5
C-5 -T-1	6	CPB/A	1:100	120, 30	Lys 1.0
C-5 -T-2	7	CPB/A	1:100	120, 120	Lys 1.0
C-5	7	CPA	1:100	15	Leu 0.6, Ile 0.7
C-6	8	APM	1:100	60	Ala 0.6
C-7	12,5	CPA	1:100	90	Trp 0.8
C-8	10 10	CPA APM	1:100 1:100	30 2	Leu 0.9 Lys 0.6, Glu 0.3, Leu 0.3
C-11	4.6	APM	1:50	120	Ala 0.4
C-12	4	CPA	1:100	20	Tyr 0.4
C-13	8 7	CPA APM	1:100 1:50	15 60	Tyr 1.0 Gly 0.8, Leu 0.7, Tyr 0.6
C-14	6 6	APM CPA/B	1:100 1:100	45 60	Gly 0.7 Ala 1.0, Arg 0.4
C-15	10	AAP	1:100	15	Gly 0.6
C-16	10 8	CPA AAP	1:25 1:100	50 60	Leu 0.9 (Ser, Gln) 0.2 Thr 0.8, Val 0.6, Ile 0.3
C-16 -T-3	5	CPA	1:5	210	Leu 0.6, Gln 0.2
C-17	6	CPA	1:200	15	Leu 0.7, Ile 0.4
C-22	6 6	CPA AAP	1:100 1:100	60 60	Leu 1.0, Gln 0.6 Arg 0.7, Thr 0.7
C-23	7 7	CPA AAP	1:50 1:100	60 45	Leu 1.0, (Ser, Asn) 0.6 Val 1.0
C-24	7 6	CPA AAP	1:100 1:50	60 45	His 0.7 Lys 0.7
C-26	14 14	CPA AAP	1:100 1:100	30 30	Leu 0.8, Arg 0.5 Thr 0.9, (Gly, Leu) 0.5
C-27	10 20 10	AAP APM CPA	1:100 1:100 1:100	30 30 120	Thr 1.0, Lys 0.3 Thr 1.0, Lys 1.0, Glu 0.5, Phe 0.5 Phe 0.8
C-28	12	CPA	1:100	15	Tyr 0.8
C-30	9 9	CPA AAP	1:100 1:1000	30 10	His 1.1, Ile 0.8, Ala 0.7 Leu 0.7, Ala 0.2
C-30a	9 9 9	AAP AAP CPA	1:1000 1:100 1:100	15 30 30	Ala 0.3 Ala 1.5, His 1.0, Ile 0.7 His 0.7, Ile 0.4, Ala 0.2

TABLE XIV - Digestion of chymotryptic peptides with trypsin

Digestion was carried out in 0.1 M ammonium bicarbonate, pH 8.5, 2 h, 37°C.
Peptides were separated by high voltage electrophoresis at pH 1.9 or 6.5

	Tryptic peptides from					
	C-5			C-16		
	T-1 ^a	T-2 ^b	T-3 ^c	T-1 ^d	T-2 ^e	T-3 ^f
Lys	0.9(1)	1.0(1)			1.0(1)	
Arg				1.1(1)		0.8(1)
Asp	1.0(1)				1.0(1)	
Thr				0.6(1)		
Ser	0.8(1)		0.8(1)			0.8(1)
Glu	1.1(1)	1.3(1)			2.2(2)	2.2(2)
Pro		1.1(1)				
Ala					2.1(2)	
Cys				(1)		
Val				1.0(1)		1.0(1)
Ile	0.9(1)	0.9(1)	1.6(2)	1.0(1)		
Leu			1.0(1)			1.0(1)
Charge	-	0				-

The amino acid compositions correspond to the following segments (Fig. 1)

^a167-171; ^b172-175; ^c176-179; ^d263-267; ^e268-273; ^f274-279

4. TRYPTIC PEPTIDES

Carboxymethylated mAATase was citraconylated (57) and digested with trypsin (2% by weight, 2 h at 37°C). The ensuing peptides were separated on a Sephadex G-25 column (Fig.11) into five fractions. Fraction I was deblocked (18 h in 100% formic acid at room temperature) and again digested with trypsin. The ensuing peptides were separated by ion-exchange chromatography (Fig.12). The pooled fractions were desalted on a Sephadex G-25 column in 0.1 M ammonium bicarbonate. Four peptides, T-4, T-6, T-13, and T-14 could be sequenced directly. By high voltage electrophoresis of the other fractions T-3, T-5, and T-15 could be separated.

Fraction II and III were deblocked in the same manner and separated each on the M-72 cation exchange resin (Fig.13 and 14). The pooled fractions had to be desalted on a Sephadex G-25 column in 0.1 M ammonium bicarbonate. The bars in Fig. 13 and 14 indicate fractions containing a peptide which could be sequenced without further purification. The other peptides were purified by preparative high voltage electrophoresis and thin-layer chromatography. Fraction II contained 10 tryptic peptides: T-1, T-2, T-7, T-16, T-19, T-21, T-22, T-23, T-25, and T-26. Fraction III contained 6 tryptic peptides: T-8, T-9, T-11, T-12, T-20, and T-27. The fraction containing T-12 was contaminated by a fragment of T-12, which was two amino acid residues shorter at the amino terminus and which could not be separated. The fraction contained four times more of the longer peptide and therefore could be sequenced unambiguously. Some material of fraction III was not soluble in 0.05 M pyridine acetate (starting buffer for the cation exchange chromatography) and could be removed by centrifugation; it proved to be the pure peptide 208-227 (T-17).

Fraction IV contained two peptides, which could be separated by high voltage electrophoresis at pH 1.9. One of them, T-9, was also found in fraction III, the other was T-24.

Fraction V could also be separated into two peptides by high voltage electrophoresis at pH 1.9. They were deblocked and found to correspond to T-10 and T-18.

All data of sequence work on the tryptic peptides can be found in the following Tables: amino acid compositions in Table XV, results of Edman degradation in Table XVI, and those from digestions with exo- and endopeptidases in Tables XVII and XVIII to XX.

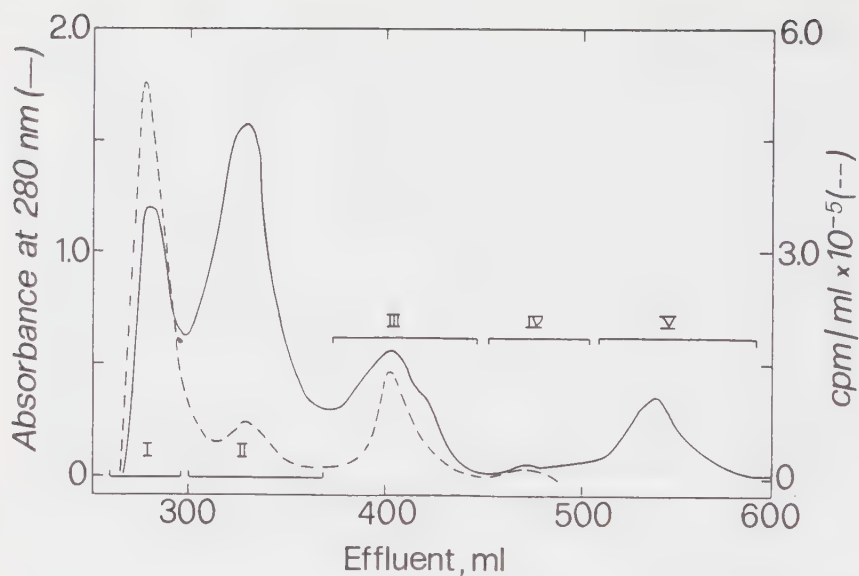


Fig. 11 - Chromatography of tryptic peptides from carboxymethylated and citraconylated mAAATase on Sephadex G-25 fine (150 x 2.6 cm-column) in 0.1 M ammonium bicarbonate, pH 8.0.

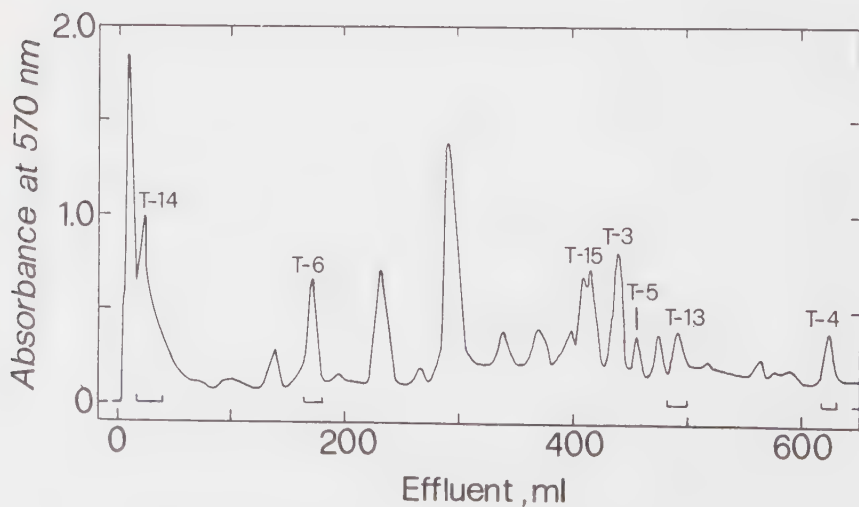


Fig. 12 - Chromatography of tryptic peptides of fraction I (Fig. 11) on a M-72 cation exchange resin as described in Fig 9.

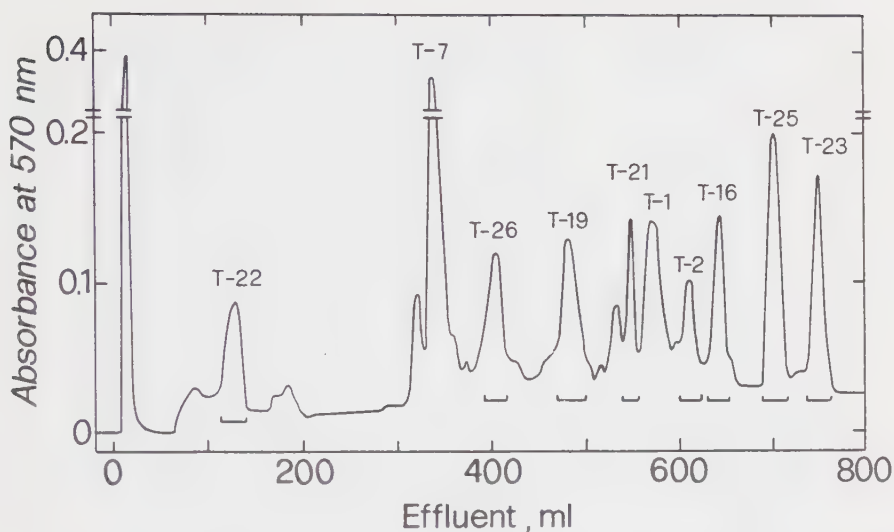


Fig. 13 - Chromatography of tryptic peptides of fraction II (Fig. 11) on a M-72 cation exchange resin as described in Fig. 9.

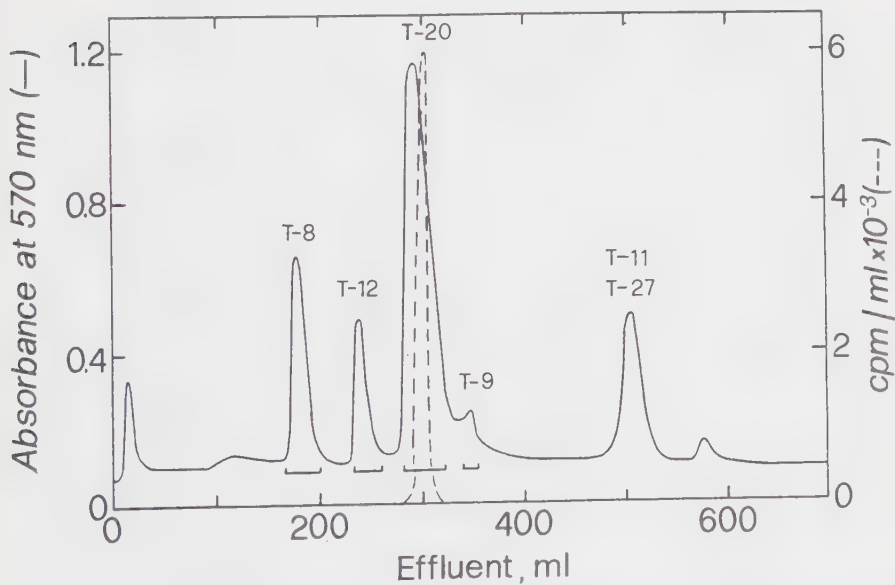


Fig. 14 - Chromatography of tryptic peptides of fraction III (Fig. 11) on a M-72 cation exchange resin as described in Fig. 9.

TABLE XV - Amino acid compositions of S-carboxymethylated and citraconylated tryptic peptides

	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10	T-11	T-12	T-13	T-14
Lys	0.9(1)	1.6(2)	1.0(1)	2.0(2)	1.0(1)		0.8(1)			0.9(1)	0.8(1)		1.0(1)	0.8(1)
His	0.6(1)										0.9(1)			
Arg	1.0(1)	1.0(1)				1.2(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)		
Asp	0.7(1)	2.6(3)		1.3(1)	1.1(1)	1.0(1)	1.5(1)		1.0(1)		1.6(2)	1.2(1)	1.1(1)	
Thr	0.9(1)	1.0(1)				0.9(1)		2.0(2)			0.9(1)			2.2(2)
Ser	2.3(3)	1.1(1)					2.4(3)	1.9(2)		0.8(1)	0.8(1)			1.6(2)
Glu	1.7(2)					0.8(1)	2.9(3)	1.3(1)	0.9(1)			1.7(2)		1.7(2)
Pro	2.2(3)					1.0(1)					2.6(3)		0.9(1)	1.3(1)
Gly	1.7(2)	2.3(2)				1.0(1)	2.1(2)	3.3(3)	1.1(1)	1.0(1)	1.0(1)	1.0(1)		1.2(1)
Ala	1.0(1)	1.1(1)	2.0(2)			1.8(2)	3.4(4)		1.0(1)			1.9(2)		1.1(1)
Cys														0.4(1)
Val	1.7(2)	1.1(1)						2.1(2)	0.8(1)		1.0(1)			
Ile	0.6(1)	1.3(1)		1.0(1)	0.5(1)						0.9(1)			0.8(1)
Met	1.0(1)		0.8(1)			1.0(1)		1.0(1)			0.9(1)			1.0(1)
Leu	1.0(1)	1.0(1)				1.8(2)	2.0(2)	1.0(1)	0.9(1)		0.8(1)	1.7(2)		1.2(1)
Tyr		1.0(1)				0.8(1)		1.1(1)			0.8(1)	0.9(1)	1.7(2)	
Phe	1.0(1)					0.9(1)	0.9(1)	0.8(1)	0.8(1)	2.5(3)	0.9(1)			1.0(1)
Trp	(2)										(1)			
Total residues	24	15	4	4	3	13	18	14	8	6	17	10	5	15
% Yield	24	45	15	18	29	28	30	76	76	38	96	96	28	44
Positions	1-24	25-39	58-61	62-65	63-65	66-78	79-96	97-110	111-118	119-124	125-141	142-151	152-156	157-171

TABLE XV continued - Amino acid compositions of S-carboxymethylated and citraconylated tryptic peptides

	T-15	T-16	T-17	T-18	T-19	T-20	T-21	T-22	T-23	T-24	T-25	T-26	T-27
Lys	0.9(1)	2.8(3)			1.0(1)		1.0(1)		1.6(2)		1.8(2)	1.0(1)	1.0(1)
His					0.8(1)							1.1(2)	1.0(1)
Arg		1.0(1)	1.0(1)	1.0(1)	0.7(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	0.6(1)		
Asp		4.3(4)	0.9(1)	0.9(1)	2.2(2)		1.0(1)	1.3(1)	1.0(1)		1.0(1)	1.4(1)	
Thr						1.0(1)		0.9(1)			1.6(2)	0.5(1)	1.0(1)
Ser		0.9(1)	1.1(1)		1.5(2)			1.1(1)		1.0(1)	0.8(1)	2.2(3)	
Glu	1.0(1)	3.6(4)	1.2(1)		4.0(4)		2.0(2)	1.4(1)	2.0(2)		1.3(1)	1.6(1)	1.2(1)
Pro	1.0(1)							1.1(1)					
Gly			2.3(2)		2.5(3)	1.1(1)			1.2(1)		0.8(1)	1.5(2)	
Ala		1.2(1)	3.2(3)	1.9(2)	1.2(1)	1.9(2)	1.8(2)	1.3(1)	1.0(1)			2.3(4)	0.9(1)
Cys						0.7(1)							
Val		1.8(2)			2.0(2)	1.0(1)			1.9(2)			2.7(4)	1.1(1)
Met			1.0(1)		1.2(1)				0.8(1)	1.1(1)	1.3(1)		
Ile			1.0(1)		1.8(2)	0.9(1)		2.0(2)		1.5(2)	0.8(1)	1.6(2)	1.0(1)
Leu	0.7(1)	1.0(1)	2.0(2)	1.0(1)	1.9(2)			3.1(3)	1.0(1)		0.9(1)	1.2(1)	
Tyr			2.2(2)		1.4(2)						0.7(1)	0.8(1)	
Phe			2.0(2)		0.9(1)	0.9(1)					0.9(1)		
Trp		(1)		1.0(1) ^a					(1)				
Total residues	4	14	20	6	25	9	7	12	13	5	14	23	7
% Yield ^b	28	76	84	65	49	91	33	73	42	48	71	53	30
Charge ^b	0						-	0					
Positions	172-175	194-207	208-227	228-233	234-258	259-267	268-274	297-308	309-321	322-326	365-378	379-401	395-401

^a Determined by amino acid analysis in 6 N HCl containing 2% mercaptoacetic acid^b As derived from electrophoretic mobility at pH 6.5

TABLE XVI - Edman degradation of tryptic peptides

Peptide	Amount nmol	Edman reagent	Solvent	Edman degradation	Results ^a
T-1	600	PITC	DMBA	automated liquid phase	SSW →
T-2	300	PITC	DMBA	automated liquid phase	DINSKKMNLGVGAY →
T-6		DABITC	pyridine	manual	EYLP IAGLADF →
T-7	550	PITC	DMBA	automated liquid phase	ASAEALGENSEAFKSG →
T-8	44	DABITC	pyridine	manual	YVTVQGI →
T-8	36	DABITC	Quadrrol Polybrene	automated liquid phase	YVTVQGISG →
T-9	820	PITC	DMBA	automated liquid phase	VGANFLQ →
T-10	750	PITC	DMBA	automated liquid phase	FFKF →
T-11	950	PITC	DMBA	automated liquid phase	DVYLPKPSWGNGHTPIF →
T-12	30	DABITC	Quadrrol	automated liquid phase	DAGLELQAY →
T-13	44	DABITC	pyridine	manual	GLELQA →
T-16	400	PITC	DMBA	automated liquid phase	YVD →
T-17	105	PITC	NMM	automated solid phase	EQWKELA →
T-17	275	PITC	DMBA	automated liquid phase	NLLAYFDM →
					NLLAYFDMAYQGFA →

T-17-C-3	62	DABITC	pyridine	manual	A S G D I N
T-18	766	PITC	DMBA	automated liquid phase	D A W A L
T-19	21	PITC	pyridine	manual	H F I E Q G I D V
T-19	30	DABITC	Quadrol	automated liquid phase	H F I E Q G I D V V L S Q S Y
T-20	870	PITC	DMBA	automated liquid phase	A G A F T V I C
T-21	140	DABITC	NMM	automated solid phase	D A E E A
T-21	15	DABITC	pyridine	manual	D
T-22	350	PITC	DMBA	automated liquid phase	I A S L I L N T P E L
T-23	280	PITC	DMBA	automated liquid phase	K E W L Y E
T-25	370	PITC	DMBA	automated liquid phase	L T K E
T-25-C-2	35	DABITC	pyridine	manual	S I Y
T-26	35	DABITC	Quadrol	automated liquid phase	I S V A G V A S S N V G Y
T-27	950	PITC	DMBA	automated liquid phase	A I H Q V I K

^a Identification by HPLC

➤ Identification by amino acid analysis on Durrum analyzer

➤ Identification by thin-layer chromatography on silica gel

➤ Identification by gas liquid chromatography

com [¹⁴C] Carboxymethylated cysteine identified by its radioactivity

Repetitive yields were 92 to 96%

TABLE XVIII - Exopeptidase digestion of tryptic peptides
Digestions were performed under the conditions described in Table IX

Peptide	Amount nmol	Enzyme	Molar ratio	Time min	Residues released mol/mol
T-1	8 8	AAP CPB/A	1:100 1:100	30 120 30	Ser 0.6 Arg 1.0, Lys 0.8, Phe 0.6 Ala 0.2
T-2	10	CPB/A	1:100	45	Arg 0.9, Tyr 0.9
T-3	6 5	AAP CPB/A	1:100 1:100	15 120, 10	Ile 0.6, Ala 0.5 Lys 1.0, Ala 0.6
T-4	4 4	CPB/A APM	1:100 1:100	120, 60 30	Lys 0.7 Lys 0.9, Met 0.9
T-5	5 5	APM CPB/A	1:50 1:100 1:50	90 120, 60	Met 0.4, Asp 0.4, Lys 0.2 Lys 0.5
T-6	6	CPB/A	1:100	120, 10	Arg 1.0, Thr 0.8, Phe 0.8
T-7	10 10	AAP CPB/A	1:100 1:100	30 120, 60	Ala 1.3, Ser 1.0 Arg 1.0
T-7-T-1	11	CPB/A	1:100	120, 45	Lys 0.6, Phe 0.5
T-7-T-2	4	APM	1:100	60	Ser 0.9, Gly 0.7, Arg 0.4
T-8	8	AAP	1:100	30	Tyr 0.4, Val 0.3
T-9	3 3	AAP CPB/A	1:50 1:100	30 120, 30	Val 0.6 Arg 0.8, Orn 0.7, Leu 0.7, Phe 0.5
T-10	15 7	AAP CPB/A	1:100 1:100	30 60, 2	Phe 1.8, Lys 0.5 Arg 0.8, Ser 0.8, Phe 0.7, Lys 0.4
T-12	7 7	CPB/A CPB/A	1:100 1:50	120, 15 120, 15	Arg 0.9, Tyr 0.8 Arg 1.0, Tyr 1.0, Ala 0.7, Gln 0.4, Leu 0.2
	13	CPY	1:20	35	Arg 1.0, Tyr 0.6, Ala 0.6 (Gln, Leu, Glu 0.3)

T-13	4	CPY	1:20	180	Lys 0.9, Pro 0.9, Asp 0.8
	4	APM	1:100	60	Tyr 1.0
T-14	3	AAP	1:100	30	Thr 0.7
T-14-CN8r-2	3	CPB/A	1:50	120, 60	Lys 0.8, Ser 0.7
T-16-T-1	9	CPB/A	1:50 1:100	120, 90	Lys 0.9, Trp 0.8 Gln 0.8
T-16-T-2	9	CPB/A	1:50 1:100	120, 90	Lys 1.0, Val 1.8, Ser 0.4
T-17-CN8r-2	4	AAP	1:100	20	Ala 0.4, Tyr 0.2, Gln 0.1
	7	CPB/A	1:50 1:100	60, 60	Arg 1.0, Asn 1.0
T-17-CN8r-2-C-1	2.3	CPA	1:100	45	Phe 1.0, Gly 1.0, Gln 1.0
T-18	7	CPB/A	1:100	120, 10	Arg 1.0, Leu 1.0, Ala 1.0, Trp 0.6
T-19, C-3	4.5	APM	1:100	90	Gly 0.7, (Glu 0.4, Arg 0.4)
T-19, C-2	1.7	CPA	1:100	30	Tyr 0.5, Leu 0.4
T-20	9	CPB/A	1:100 1:50	120, 60	Arg 1.0
	9	AAP	1:100	30	Ala 1.1, Gly 0.5
T-21	10	CPB/A	1:100	120, 90	Arg 1.0, Lys 0.8, Ala 0.4
T-22	7	CPB/A	1:100	120, 30	Arg 1.0, Leu 1.0
	7	AAP	1:100	15	Ile 1.2, Ala 0.5, Ser 0.4, Leu 0.4
T-23-T-2	2.5	APM	1:50	30	Gly 1.0, Met 0.6, (Ala 0.5, Asp 0.5)
T-23-T-1	9	CPB/A	1:100 1:50	120, 180	Lys 1.0(Val 1.9, Glu 0.9)
T-24	6	CPB/A	1:100	120, 10	Arg 1.0, Met 0.8
	6	AAP	1:100	20	Ile 1.8, Ser 0.8, Met 0.8
T-25	8	APM	1:50	60	Leu 0.7, Thr 0.6, Lys 0.5
T-25-C-2	4	AAP	1:100	20	Ser 0.7
T-26	7	CPB/A	1:100	120, 15	Lys 0.8, Thr 0.5
T-27	10	AAP	1:100	60	Ala 0.6, Ile 0.5, His 0.5

TABLE XVIII - Digestion of tryptic peptides with deblocked lysine residues with trypsin
Digestion was carried out in 0.1 M ammonium bicarbonate, pH 8.5, 3 h, 37° C. Peptides
were separated by high voltage electrophoresis at pH 1.9 or 6.5

	Tryptic peptides from						
	T-7		T-16			T-23	
	T-1 ^a	T-2 ^b	T-1 ^c	T-2 ^d	T-3 ^e	T-1 ^f	T-2 ^g
Lys	1.0(1)		1.0(1)	1.0(1)	0.9(1)	2.0(2)	1.0(1)
Arg		1.0(1)			1.0(1)		1.0(1)
Asp	1.2(1)			0.7(1)			
Ser	1.9(2)	0.7(1)		1.2(1)		2.0(2)	
Glu	2.8(3)		3.3(3)				
Gly	1.2(1)	1.0(1)		1.0(1)			1.0(1)
Ala	3.8(4)			2.0(2)		2.2(2)	0.9(1)
Val							
Met	2.1(2)			0.9(1)		1.0(1)	
Leu	1.0(1)						
Phe							
Trp			(1)			(1)	

The amino acid compositions correspond to the following segments Fig. 1):

^a 79-93; ^b 94-96; ^c 194-198; ^d 199-205; ^e 206-207; ^f 309-316; ^g 317-321

TABLE XIX - Digestion of tryptic peptides with chymotrypsin

Digestion was carried out in 0.1 M ammonium bicarbonate, pH 8.5, 3 to 4 h, 37°C. Peptides were separated by high voltage electrophoresis at pH 1.9 or 6.5

Chymotryptic peptides from												
T-17	T-17	T-17	T-17	T-17-CNBr-2	T-19	T-19	T-19	T-25	T-25	T-25	T-26	T-26
C-1 ^a	C-2 ^b	C-3 ^c	C-1 ^d	C-2 ^e	C-1 ^f	C-2 ^g	C-3 ^h	C-1 ⁱ	C-2 ^k	C-3 ^l	C-1 ^m	C-2 ⁿ
C-3 ^o												
Lys					0.4(1)	0.8(1)	1.0(1)	1.0(1)		1.0(1)		2.0(2)
His												
Arg		1.0(1)		1.0(1)								
Asp	0.9(1)	1.0(1)	1.6(2)	1.9(2)	1.1(1)	1.0(1)	1.0(1)	0.8(1)		0.9(1)	1.4(1)	0.9(1)
Thr												
Ser		0.7(1)		0.8(1)	1.2(2)			1.1(1)	0.8(1)		2.5(3)	
Glu			1.2(1)		2.6(3)		1.2(1)					
Gly		1.0(1)	1.1(1)	0.9(1)	0.8(1)	0.9(1)	0.9(1)			1.1(1)	2.4(2)	1.0(1)
Ala	1.1(1)	1.1(1)	0.7(1)	1.0(1)	1.6(2)	1.0(1)					2.0(2)	2.1(2)
Val											3.2(3)	
Met		1.0(1)				0.7(1)				0.7(1)	1.0(1)	1.0(1)
Ile		0.9(1)		1.0(1)	1.4(2)			0.7(1)	1.0(1)			0.8(1)
Leu	1.4(2)				1.1(1)	1.0(1)						
Tyr	1.0(1)	1.0(1)			1.0(1)	0.8(1)			1.0(1)		0.9(1)	
Phe	1.1(1)			0.9(1)	0.7(1)			0.8(1)				

The amino acid compositions correspond to the following segments (Fig. 1):

a²⁰⁸⁻²¹³; b²¹⁴⁻²¹⁷; c²²¹⁻²²⁷; d²¹⁶⁻²²⁰; e²²¹⁻²²⁷; f²³⁴⁻²⁴⁸; g²⁴⁹⁻²⁵⁵; h²⁵⁶⁻²⁵⁸; i³⁶⁵⁻³⁶⁹; k³⁷⁰⁻³⁷²; l³⁷³⁻³⁷⁸; m³⁷⁹⁻³⁹¹; n³⁹²⁻³⁹⁷; o³⁹⁸⁻⁴⁰¹

TABLE XX - Cleavage of tryptic peptides

Cleavage with CNBr was carried out in 70% formic acid, 24 h, room temperature. Peptides were separated by high voltage electrophoresis at pH 1.9 or by thin-layer chromatography. Cleavage at aspartic acid residues in 0.25 M acetic acid was performed at 106° C in evacuated sealed Pyrex tubes for 18 h (58). Peptides were separated by high voltage electrophoresis at pH 6.5

	Cyanogen bromide peptides from				Peptides of cleavage in acetic acid from	
	T-14		T-17		T-14	
	CNBr-1 ^a	CNBr-2 ^b	CNBr-1 ^c	CNBr-2 ^d	Ac-1 ^e	Ac-2 ^f
Lys		0.7(1)				1.0(1)
Arg				1.0(1)		
Asp	1.2(1)	1.0(1)	1.9(2)	2.2(2)	1.0(1)	
Thr	0.9(1)					
Ser	0.8(1)	0.9(1)		1.0(1)		0.9(1)
Glu	0.8(1)	1.0(1)		1.0(1)		
Gly	1.0(1)			2.2(2)		
Ala	0.8(1)		1.0(1)	2.0(2)		
Cys	(1)					
Met	0.4(1)		0.8(1)			
Ile		0.9(1)		1.0(1)		1.0(1)
Leu	1.0(1)		1.8(2)			
Tyr			0.7(1)	0.8(1)		
Phe	0.8(1)		0.8(1)	0.9(1)		

The amino acid compositions correspond to the following segments (Fig. 1):

^a157-166; ^b167-171; ^c208-215; ^d216-227; ^e168; ^f169-171

5. PEPTIDES FROM DIGESTION WITH STAPHYLOCOCCUS AUREUS PROTEASE V8

Aminoethylated mAATase (59) was digested with Staphylococcus aureus protease V8 (4% by weight, 0.1 M ammonium bicarbonate, pH 7.8, 18 h, 37° C). Fig.15 shows the separation of the peptides on Sephadex G-50. Fraction II and III were pooled and separated on M-72 cation exchange resin (Fig.16). Peptides which could be sequenced without further purification are indicated by bars (Fig. 16), the others were purified by high voltage electrophoresis. Ten of the peptides obtained by digestion with Staphylococcus aureus protease were utilized, their amino acid compositions are given in Table XXI, data on Edman degradation in Table XXII, and from exo- and endopeptidase digestions in Tables XXIII and XXIV.

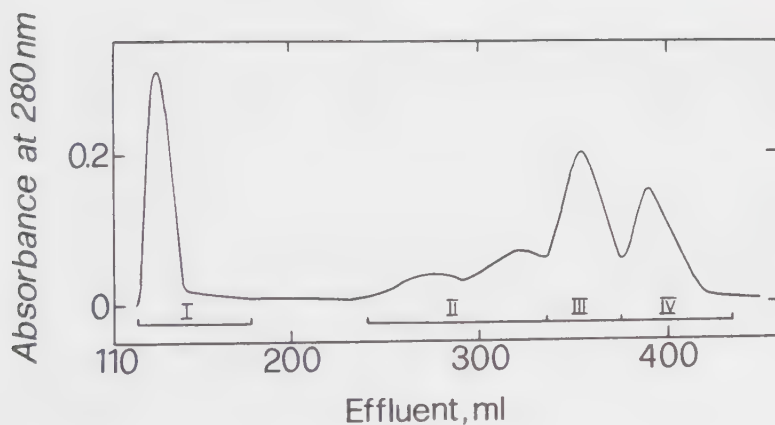


Fig. 15 - Chromatography of peptides obtained by digestion of aminoethylated mAATase with Staphylococcus aureus protease V8 on Sephadex G-50 (120 x 2.6 cm-column) in 0.1 M ammonium bicarbonate.

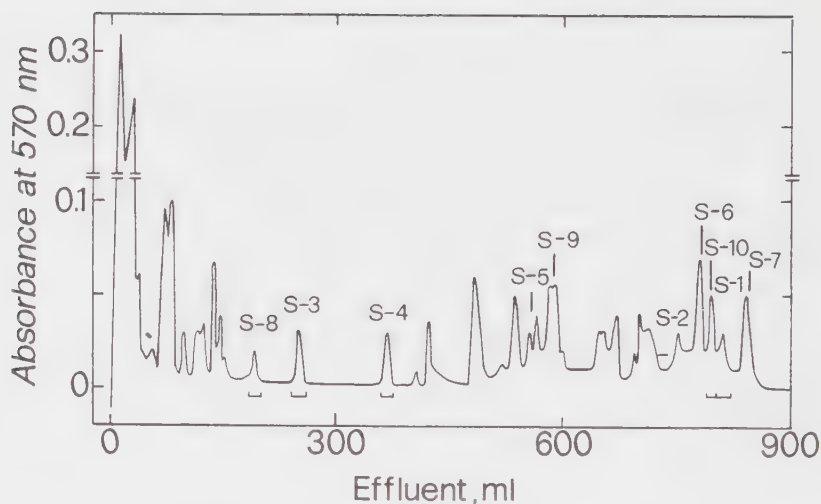


Fig. 16 - Chromatography of pooled fraction II and III obtained by digestion with *Staphylococcus aureus* protease V8 (Fig. 15) on a M-72 cation exchange resin as described in Fig. 9.

TABLE XXI - Amino acid compositions of peptides from digestion of aminoethylated mAATase with *Staphylococcus aureus* protease V8

	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10
Lys	1.1(1)	1.2(1)	1.0(1)	1.0(1)		1.0(1)	1.0(1)			1.0(1)
His										
Arg	1.0(1)	1.2(1)			0.8(1)	0.7(1)	1.0(1)		1.6(2)	0.9(1)
Asp	1.0(1)	2.2(2)	0.8(1)		1.0(1)				1.8(2)	
Thr		1.0(1)			0.7(1)				0.9(1)	0.9(1)
Ser		1.1(1)	0.9(1)	0.9(1)					1.8(2)	
Glu		0.8(1)	1.2(1)	1.2(1)	1.8(2)	1.1(1)	1.1(1)	1.0(1)	1.0(1)	1.0(1)
Pro		1.0(1)	1.1(1)	1.0(1)						
Gly										
Ala	0.9(1)	1.0(1)			1.0(1)	0.8(1)			0.7(1)	
Cys		0.6(1)			0.7(1)					
Val					0.6(1)	0.8(1)		0.9(1)		
Ile			1.8(2)	1.9(2)	0.8(1)				0.9(1)	
Leu		1.4(2)					0.8(1)	0.9(1)		0.8(1)
Tyr		2.3(3)								
Phe	0.7(1)									
Trp								(1)	(1)	
Total residues	5	15	7	6	9	5	4	4	11	5
% Yield	40	7	14	66	5	3	6	27	48	42
Charge	+		-	0		+				
Position	21- 25	147-161	168-174	169-174	263-271	272-276	307-310	311-314	337-347	364-368

TABLE XXII - Edman degradation of peptides from digestion of aminoethylated mAATase with *Staphylococcus aureus* protease V8

Peptide	Amount	Edman reagent	Solvent	Edman degradation	Results ^a
S-1	70	PITC	pyridine	manual	<u>A</u> <u>F</u>
S-4	35	PITC	pyridine	manual	<u>I</u> <u>S</u>
S-7	59	PITC	pyridine	manual	<u>L</u> <u>R</u>
S-9	21	DABITC	Quadrol + Polybrene	automated liquid phase	G S S H N W Q H I T → → → → → → → →
S-10	64	PITC	pyridine	manual	<u>R</u> <u>L</u>

^a → Identification by HPLC
→ Identification by amino acid analysis on Durrum analyzer
Repetitive yields were 92 to 95%

TABLE XXIII - Exopeptidase digestion of peptides from digestion of aminoethylated mAATase with *Staphylococcus aureus* protease V8

Digestions were performed as described in Table IX

Peptide	Amount nmol	Enzyme	Molar ratio	Time min	Residues released mol/mol
S-2	12	CPY/A	1:17, 1:10	60, 60	Asp 1.0, Leu 1.0, Ser 0.8, (AE-Cys 0.3, Thr 0.4)
S-2-T-3	9	CPY	1:17	60	Asp 1.0, Leu 1.0
S-6	7	AAP	1:50	60	Ala 0.9, Lys 0.8, Arg 0.7
S-9	9	CPY	1:17	15	Asp 1.0, Thr 0.6, Ile 0.4

TABLE XXIV - Tryptic digestion of peptide S-2 obtained by cleavage of aminoethylated mAATase with *Staphylococcus aureus* protease V8

Digestion was performed as described in Table IX

Tryptic peptides from S-2				
	T-1 ^a	T-2 ^b	T-3 ^c	T-4 ^d
Lys		0.9(1)		
Arg	1.0(1)			
Asp		1.2(1)		1.2(1)
Thr			1.0(1)	
Ser				1.0(1)
Pro		1.0(1)		
Ala	1.0(1)			
Cys			1.0(1)	
Leu				1.0(1)
Tyr	1.0(1)	1.8(2)		

The amino acid compositions correspond to the following segments (Fig. 1):

^a 149-151; ^b 152-156; ^c 157-158; ^d 159-161

6. TRYPTIC PEPTIDES OF NEM-MODIFIED mAATase

mAATase was modified with N- 2,3- $[^{14}\text{C}]$ ethylmaleimide (NEM¹, Amersham) (60) and digested with trypsin (6% by weight, 2 h, 37°C). After chromatography on a Sephadex G-25 column (Fig.17) the three main radioactive fractions were pooled and subjected to a diagonal procedure for isolation of sulfhydryl peptides alkylated with NEM (19). Four of the five sulfhydryl peptides could be isolated by this procedure. Fraction I contained NEM-T-2 and NEM-T-3, both of them could be sequenced by the automated procedure. Fraction II contained NEM-T-5, only the amino acid composition is given. Fraction III contained NEM-T-1, which was sequenced manually, and NEM-T-4, of which only the amino acid composition is given. NEM-T-4 is one of the numerous by-products formed by the action of chymotrypsin contaminating the preparation of trypsin used, in spite of the use of TPCK-trypsin. The low yields of the separated peptides may also be due to chymotryptic activity, there were many spots outside the diagonal which could not be separated. The amino acid compositions of the five NEM-peptides are given in Table XXV, the data on their Edman degradation in Table XXVI.

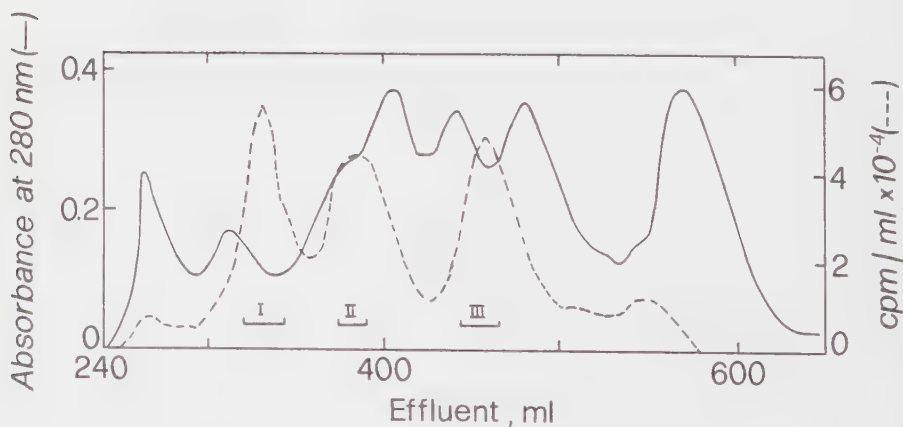


Fig. 17 - Chromatography of tryptic peptides of NEM-modified mAATase on Sephadex G-25 (150 x 2.6 cm-column) in 10% acetic acid.

TABLE XXV - Amino acid compositions of tryptic NEM-peptides of NEM-modified mAATase

	NEM-T-1	NEM-T-2	NEM-T-3	NEM-T-4	NEM-T-5
Lys		1.9(2)			0.8(1)
His			1.5(2)		
Arg	0.8(1)		1.2(1)	0.9(1)	1.0(1)
Asp	1.1(1)	2.1(2)	1.7(2)	3.4(4)	
Thr		1.7(2)	0.8(1)		0.6(1)
Ser		1.7(2)	0.8(1)	0.9(1)	
Glu		2.1(2)		1.2(1)	3.2(3)
Pro		1.0(1)	1.5(2)		1.0(1)
Gly		1.4(1)	1.3(1)	1.8(2)	1.4(1)
Ala		1.4(1)	2.0(2)	2.6(3)	
Cys	(1)	(1)	(1)		(1)
Val	1.6(2)		1.0(1)		0.6(1)
Met		0.8(1)		1.0(1)	
Ile		1.9(2)	1.5(2)	1.1(1)	
Leu	1.0(1)	1.0(1)	1.7(2)	1.9(2)	1.1(1)
Tyr				1.6(2)	
Phe		1.0(1)		1.7(2)	0.9(1)
Total residues	6	19	18	20	12
% Yield	6	8	16	8	8
Position	47- 52	157-175	176-193	208-227	353-364

TABLE XXVI - Edman degradation of tryptic NEM-peptides of NEM-modified mAATase

Peptide	Amount nmol	Edman reagent	Solvent	Edman degradation	Results ^a
NEM-T-1	16	DABITC	pyridine	manual	V L N C →→→cpm
NEM-T-2	18	DABITC	Quadrol + Polybrene	automated liquid phase	T C S L D F T G A M E D →→→→→→→→→cpm
NEM-T-3	16	DABITC	Quadrol + Polybrene	automated liquid phase	S I I L L H A C A H N P T G V D P →→→→→→→→→cpm

^a → Identification by HPLC

→ Identification by amino acid analysis on Durrum analyzer

cpm [¹⁴C] NEM-modified cysteine identified by its radioactivity
Repetitive yields were 93 to 98%

TABLE XXVII - Edman degradation of carboxymethylated mAAATase

The amino acid sequence of residue 1 through 40 was determined by automated Edman degradation of carboxymethylated mAAATase (CM-mAAATase) on a liquid phase sequencer. Repetitive yield was between 94% (Val 9 - Val 20) and 97% (Arg 24 - Arg 39).

Peptide	Amount	Edman reagent	Solvent	Result ^a
CM-mAAATase	400	PITC	DMBA	S S W W S H V E M G P P D P I L G V T E A F K R D T N S K K M N L G V G A Y R D

^a → Identification by amino acid analysis on Durrum analyzer
→ Identification by gas liquid chromatography

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Winterthur, den 30.8.1981

Lebenslauf

Ursula Elisabeth Graf-Hausner von Bärethswil (ZH), geboren am 21.9.1951 in Mainz-Gustavsburg (Bundesrepublik Deutschland).

1958 - 1962 Absolvieren der Volksschule in Ludwigshafen/Rhein und Düsseldorf.

1962 - 1965 Besuch des altsprachlichen Luisengymnasiums in Düsseldorf.

1965 - 1971 Ausbildung am humanistischen Th.-Heuss-Gymnasium in Ludwigshafen und Abschluss mit dem alt- und neusprachlichen Abitur.

1971 - 1976 Studium mit Biologie und Chemie im Hauptfach an der Universität Heidelberg.

1975 - 1976 Ausführung der wissenschaftlichen Arbeit am Biochemischen Institut der Universität Zürich bei Prof. Dr. Ph. Christen.

Nov. 1976 Staatsexamen in Biologie und Chemie an der Universität Heidelberg.

1977 - 1981 Assistent am Biochemischen Institut der Universität Zürich und Ausführung der Dissertation unter der Leitung von Prof. Dr. Ph. Christen.

Bei den folgenden Dozenten der Universität Zürich besuchte ich Vorlesungen, Uebungen und Kurse:

Baumgartner, Biegert, Billeter, Birnstiel, Bosshard, Chen, Christen, Greeff, Greuter, Humbel, Kägi, Kiechler, Kubik, Lindenmann, Nöthiger, Torr, Uebersax-Ridley, Woodtli.

